PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/16, 15/31, 15/62, 1/21, C07K 14/285, 7/23, A61K 38/09, 39/385 — A1

(11) International Publication Number: **WO 98/06848**

(43) International Publication Date: 19 February 1998 (19.02.98)

(21) International Application Number: PCT/CA97/00559

(22) International Filing Date: 8 August 1997 (08.08.97)

(30) Priority Data:
08/694,865 9 August 1996 (09.08.96) US

(71) Applicant: UNIVERSITY OF SASKATCHEWAN [CA/CA]; 124 Veterinary Road, Saskatoon, Saskatchewan S7N 0W0 (CA).

(72) Inventors: POTTER, Andrew, A.; 521 Dalhousie Crescent, Saskatoon, Saskatchewan S7H 3S5 (CA). MANNS, John, G.; 11 Pony Trail Road, Riverside Estate, Saskatoon, Saskatchewan S7T 1A1 (CA).

(74) Agents: ERRATT, Judy, A. et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GnRH-LEUKOTOXIN CHIMERAS

GnRH-1:

```
          Gln His Trp Ser Tyr Gly Leu Arg Pro Gly
...CAG CAT TGG AGC TAC GGC CTG CGC CCT GGC...
...GTC GTA ACC TCG ATG CCG GAC GCG GGA CCG...
```

A

GnRH-2:

```
          (1)                                                                    (2)
       (Gln His Trp Ser Tyr Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser
...CAG CAT TGG AGC TAC GGC CTG CGC CCT GGC AGC GGT TCT CAA GAT TGG AGC
...GTC GTA ACC TCG ATG CCG GAC GCG GGA CCG TCG CCA AGA GTT CTA ACC TCG
   1                  5                  10                  15

                                                    (3)
Tyr Gly Leu Arg Pro Gly Gly Ser Ser Gln His Trp Ser Tyr Gly Leu Arg
TAC GGC CTG CGT CCG GGT GGC TCT AGC CAG CAT TGG AGC TAC GGC CTG CGC
ATG CCG GAC GCA GGC CCA CCG AGA TCG GTC GTA ACC TCG ATG CCG GAC GCG
            20                  25                  30

                                  (4)
Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro Gly],
CCT GGC AGC GGT AGC CAA GAT TGG AGC TAC GGC CTG CGT CCG GGT...
GGA CCG TCG CCA TCG GTT CTA ACC TCG ATG CCG GAC GCA GGC CCA...
 35                  40                  45                  49
```

B

(57) Abstract

New immunological carrier systems, DNA encoding the same, and the use of these systems, are disclosed. The carrier systems include chimeric proteins which include a leukotoxin polypeptide fused to one or more selected GnRH multimers which comprise at least one repeating GnRH decapeptide sequence, or at least one repeating unit of a sequence corresponding to at least one epitope of a selected GnRH molecule. Under the invention, the selected GnRH sequences may all be the same, or may correspond to different derivatives, analogues, variants or epitopes of GnRH so long as the GnRH sequences are capable of eliciting an immune response. The leukotoxin functions to increase the immunogenicity of the GnRH multimers fused thereto.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

GnRH-LEUKOTOXIN CHIMERAS

Description

Technical Field

The present invention relates generally to immunological carrier systems. More particularly, the invention pertains to leukotoxin-GnRH chimeras including more than one copy of a GnRH polypeptide. The chimeras demonstrate enhanced immunogenicity as compared to the immunogenicity of GnRH polypeptides alone.

Background of the Invention

In vertebrates, synthesis and release of the two gonadotrophic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are regulated by a polypeptide referred to as Gonadotropin releasing hormone (GnRH) (formerly designated LHRH). Accordingly, one approach to fertility control in an animal population is to reduce the levels of GnRH, such as by immunization against GnRH, which effects a reduction in the levels of LH and FSH and the concomitant disruption of estrous cycles and spermatogenesis. See e.g., Adams et al., *J. Anim. Sci.* (1990) 68:2793-2802.

Early studies of the GnRH molecule have shown that it is possible to raise antisera in response to repeated injections of synthetic GnRH peptides (Arimura et al., *Endocrinology* (1973) 93(5):1092-1103). Further, antibodies to GnRH have

been raised in a number of species by chemical conjugation of GnRH to a suitable carrier and administration of the conjugate in an appropriate adjuvant (Carelli et al., *Proc. Natl. Acad. Sci.* (1982) 79:5392-5395). Recombinant fusion proteins comprising GnRH or GnRH-analogues have also been described for use in peptide vaccines for the immunological castration or inhibition of reproductive function of various domesticated and farm animals (Meloen et al., *Vaccine* (1994) 12(8):741-746; Hoskinson et al., *Aust. J. Biotechnol.* (1990) 4:166-170; and International Publication Nos. WO 92/19746, published 12 November 1992; WO 91/02799, published 7 March 1991; WO 90/11298, published 4 October 1990 and WO 86/07383, published 18 December 1986).

However, attempts have fallen short of providing adequate immunological sterilization products due to the poor immunogenicity of GnRH peptides and due to the fact that chemical conjugation protocols are difficult to control, rendering substantially heterogenous and poorly-defined GnRH conjugates. Further, peptide vaccines based on GnRH have met with limited success in providing uniform effects on individual animal subjects even after repeated vaccination. In this regard, prior GnRH constructs have failed to provide a uniformly successful immunological sterilization vaccine product due to the fact that GnRH is a small, "self" molecule that is not normally recognized by a subject's immune system, rendering the molecule poorly immunogenic and inherently unable to induce a significant immune response against endogenous GnRH.

It is generally recognized that the immunogenicity of viral antigens, small proteins or endogenous substances may be significantly increased by producing immunogenic forms of those molecules

comprising multiple copies of selected epitopes. In this regard, constructs based on two or four repeats of peptides 9-21 of herpes simplex virus type 1 glycoprotein D (Ploeg et al., *J. Immuno. Methods* (1989) 124:211-217), two to six repeats of the antigenic circumsporozoite tetrapeptide NPNA of *Plasmodium falciparum* (Lowell et al., *Science* (1988) 240:800-802), two or four copies of the major immunogenic site of VP1 of foot-and-mouth disease virus (Broekhuijsen et al., *J. gen. Virol.* (1987) 68:3137-3143) and tandem repeats of a GnRH-like polypeptide (Meloen et al., *Vaccine* (1994) 12(8):741-746), have been shown to be effective in increasing the immunogenicity of those molecules.

Small proteins or endogenous substances may also be conjugated to a suitable carrier in order to elicit a significant immune response in a challenged host. Suitable carriers are generally polypeptides which include antigenic regions of a protein derived from an infectious material such as a viral surface protein, or a carrier peptide sequence. These carriers serve to non-specifically stimulate T helper cell activity and to help direct antigen to antigen presenting cells for processing and presentation of the peptide at the cell surface in association with molecules of the major histocompatibility complex (MHC).

Several carrier systems have been developed for this purpose. For example, small peptide antigens are often coupled to protein carriers such as keyhole limpet haemocyanin (Bittle et al., *Nature* (1982) 298:30-33), tetanus toxoid (Muller et al., *Proc. Natl. Acad. Sci. U.S.A.* (1982) 79:569-573), ovalbumin, and sperm whale myoglobin, to produce an immune response. These coupling reactions typically result in the incorporation of several moles of peptide antigen per

mole of carrier protein. Although presentation of the peptide antigen in multiple copies generally enhances immunogenicity, carriers may elicit strong immunity not relevant to the peptide antigen and this may inhibit the immune response to the peptide vaccine on secondary immunization (Schutze et al, *J. Immun.* (1985) 135:2319-2322).

Antigen delivery systems have also been based on particulate carriers. For example, preformed particles have been used as platforms onto which antigens can be coupled and incorporated. Systems based on proteosomes (Lowell et al., *Science* (1988) 240:800-802), immune stimulatory complexes (Morein et al., *Nature* (1984) 308:457-460), and viral particles such as HBsAg (Neurath et al., *Mol. Immunol.* (1989) 26:53-62) and rotavirus inner capsid protein (Redmond et al., *Mol. Immunol.* (1991) 28:269-278) have been developed.

Carrier systems have also been devised using recombinantly produced chimeric proteins that self assemble into particles. For example, the yeast retrotransposon, Ty, encodes a series of proteins that assemble into virus like particles (Ty-VLPs; Kingsman, S. M., and A. J. Kingsman *Vacc.* (1988) 6:304-306). Foreign genes have been inserted into the *Ty*A gene and expressed in yeast as a fusion protein. The fusion protein retains the capacity to self assemble into particles of uniform size.

Other chimeric protein particles have been examined such as HBsAg, (Valenzuela et al., *Bio/Technol.* (1985) 3:323-326; U.S. Patent No. 4,722,840; Delpeyroux et al., *Science* (1986) 233:472-475), Hepatitis B core antigen (Clarke et al., Vaccines 88 (Ed. H. Ginsberg, et al., 1988) pp. 127-131), Poliovirus (Burke et al., *Nature* (1988) 332:81-82), and Tobacco Mosaic Virus (Haynes et al.,

Bio/Technol. (1986) 4:637-641). However, these carriers are restricted in their usefulness by virtue of the limited size of the active agent which may be inserted into the structural protein without interfering with particle assembly.

Finally, chimeric systems have been devised using a *Pasteurella haemolytica* leukotoxin (LKT) polypeptide fused to a selected antigen. See, e.g., International Publication Nos. WO 93/08290, published 29 April 1993 and WO 92/03558, published 5 March 1992, as well as U.S. Patent Nos. 5,238,823 and 5,273,889. Inclusion of a LKT carrier portion in a peptide antigen chimera supplies enhanced immunogenicity to the chimera by providing T-cell epitopes having broad species reactivity, thereby eliciting a T-cell dependent immune response in immunized subjects. In this regard, inducement of adequate T-cell help is essential in the generation of an immune response to the peptide antigen portion of the chimera, particularly where the antigen is an endogenous molecule. However, the use of a leukotoxin polypeptide carrier in combination with multiple epitopes of the GnRH peptide has not heretofore been described.

Disclosure of the Invention

The present invention is based on the construction of novel gene fusions between the *P. haemolytica* leukotoxin gene, variants thereof, and one or more nucleotide sequences encoding multiple GnRH polypeptides. These constructs produce chimeric proteins that display surprisingly enhanced immunogenicity when compared to the immunologic reaction elicited by administration of GnRH alone.

Thus in one embodiment, the present invention is directed to a chimeric protein comprising

a leukotoxin polypeptide fused to one or more multimers wherein each multimer comprises more than one selected GnRH polypeptide. The leukotoxin portion of the chimera acts to increase the immunogenicity of the GnRH polypeptides. More particularly, the GnRH multimers used herein may correspond to more than one copy of a selected GnRH polypeptide or epitope, or multiple tandem repeats of a selected GnRH polypeptide or epitope. Further, GnRH multimers may be located at the carboxyl and/or amino terminal of the leukotoxin polypeptide, at sites internal to the leukotoxin polypeptide, or any combination of such sites. Each GnRH multimer may also correspond to a molecule of the general formula GnRH-X-GnRH, wherein X is selected from the group consisting of a peptide linkage, an amino acid spacer group and $[GnRH]_n$, where n is greater than or equal to 1, and further wherein "GnRH" may comprise any GnRH polypeptide. In one particular embodiment, a chimeric protein comprising a leukotoxin polypeptide fused to two GnRH multimers is provided. In this molecule, the C-terminus of one of the GnRH multimers is fused to the N-terminus of the leukotoxin polypeptide, and the N-terminus of the leukotoxin polypeptide is fused to the N-terminus of the other GnRH multimer.

Also disclosed are vaccine compositions comprising the chimeric proteins with a pharmaceutically acceptable vehicle, as well as methods for presenting one or more selected GnRH multimers to a host subject by the administration of an effective amount of the subject vaccine compositions.

In another embodiment, the invention is directed to DNA constructs encoding the chimeric proteins. The DNA constructs comprise a first nucleotide sequence encoding a leukotoxin polypeptide

operably linked to one or more selected nucleotide sequences, each selected nucleotide sequence encoding more than one copy of a GnRH polypeptide or epitope.

In yet another embodiment, the invention is directed to expression cassettes comprised of the above-described DNA constructs operably linked to control sequences that direct the transcription thereof, whereby the constructs can be transcribed and translated in a host cell.

In another embodiment, the invention is directed to host cells transformed with these expression cassettes.

Another embodiment of the invention provides a method of producing a recombinant polypeptide. The method comprises (a) providing a population of host cells described above and (b) culturing the population of cells under conditions whereby the chimeric polypeptide encoded by the expression cassette is expressed.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figures 1A and 1B show the nucleotide sequences and amino acid sequences of the GnRH constructs used in the chimeric leukotoxin-GnRH polypeptide gene fusions. Figure 1A depicts GnRH-1 which includes a single copy of a GnRH decapeptide; Figure 1B depicts GnRH-2 which includes four copies of a GnRH decapeptide when n=1, and eight copies of GnRH when n=2, etc.

Figure 2 depicts the structure of Plasmid pAA352 wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the β-lactamase gene (ampicillin resistance); ori is the ColE1-based

plasmid origin of replication; lktA is the *P. haemolytica* leukotoxin structural gene; and lacI is the *E. coli* lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

Figures 3-1 through 3-9 show the nucleotide sequence and predicted amino acid sequence of leukotoxin 352 (LKT 352). Both the structural gene for LKT 352 and the sequences of the flanking vector regions are shown.

Figure 4 shows the structure of Plasmid pCB113 carrying a leukotoxin-GnRH (LKT-GnRH) gene fusion.

Figures 5-1 through 5-8 show the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB113. The nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB112 are identical to the sequences of the chimeric protein derived from pCB113 except that the sequence for multiple copy GnRH was inserted twice as described above in regard to Figure 4.

Figure 6 shows the structure of Plasmid pCB111 carrying a leukotoxin-GnRH (LKT-GnRH) gene fusion.

Figures 7-1 through 7-5 show the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB111. The nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB114 are identical to the sequences of the chimeric protein derived from pCB111 except that the sequence for multiple copy GnRH was inserted twice as described above in regard to Figure 6.

Figures 8-1 through 8-2 show the nucleotide sequence and predicted amino acid sequence of the blunt end fusion point of the truncated leukotoxin gene of plasmid pCB111 (Figure 8-2), where an internal DNA fragment (of approximately 1300 bp in length) was removed from LKT 352 by digestion with the restriction enzymes *Bst*B1 and *Nae*1 (Figure 8-1).

Figures 9-1 through 9-6 show the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB122.

Figure 10 shows the structure of Plasmid pAA101 carrying the LKT 101 leukotoxin polypeptide which lacks cytotoxic activity.

Figure 11 depicts the predicted amino acid sequence of the LKT 101 leukotoxin polypeptide.

Figure 12 shows a comparison of average serum anti-GnRH antibody titres in barrows, untreated boars, and immunocastrated boars (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 10.

Figure 13 shows a comparison of average serum testosterone levels in barrows, untreated boars, and immunocastrated boars (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 10.

Figure 14 shows a comparison of feed conversion efficiency (expressed as the ratio of Kg feed:Kg weight gain) in barrows, untreated boars, and immunocastrated boars (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 10.

Figure 15 shows a comparison of average serum anti-GnRH antibody titres in animals injected with a vaccine composition containing a LKT::8 copy GnRH fusion protein, or a vaccine composition containing an 8 copy GnRH::LKT::8 copy GnRH fusion protein as described in Example 11.

Figure 16 shows a comparison of average ovarian weight (mg), average uterine weight (mg), and average serum estradiol (pg/mL), in control animals (solid bars) and animals treated with a vaccine composition containing an 8 copy GnRH::LKT::8 copy GnRH fusion protein as described in Example 13 (cross-hatched bars).

Figure 17 depicts a comparison in fat androstenone levels in barrows, boars, late castrated animals, and immunocastrated animals (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 14.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual; DNA Cloning, Vols. I and II (D.N. Glover ed.) ; Oligonucleotide Synthesis (M.J. Gait ed.); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds.); Animal Cell Culture (R.K. Freshney ed.); Immobilized Cells and Enzymes (IRL press); B. Perbal, A Practical Guide to Molecular Cloning; the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications).

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The term "Gonadotropin releasing hormone" or "GnRH" refers to a decapeptide secreted by the hypothalamus which controls release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) in vertebrates (Fink, G., *British Medical Bulletin* (1979) 35:155-160). The amino acid sequence of GnRH is highly conserved among vertebrates, and especially in mammals. In this regard, GnRH derived from most mammals including human, bovine, porcine and ovine GnRH (formerly designated LHRH) has the amino acid sequence pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Murad et al., *Hormones and Hormone Antagonists*, in The Pharmacological Basis of Therapeutics, Sixth Edition (1980) and Seeburg et al., *Nature* (1984) 311:666-668).

As used herein a "GnRH polypeptide" includes a molecule derived from a native GnRH sequence, as well as recombinantly produced or chemically synthesized GnRH polypeptides having amino acid sequences which are substantially homologous to native GnRH and which remain immunogenic, as described below. Thus, the term encompasses derivatives and analogues of GnRH including any single or multiple amino acid additions, substitutions and/or deletions occurring internally or at the amino or carboxy terminuses of the peptide. Accordingly, under the invention, a "GnRH polypeptide" includes molecules having the native sequence, molecules such as that depicted in Figure 1A (having an N-terminal Gln residue rather than a pyroGlu residue), and molecules with other amino acid additions, substitutions and/or deletions which retain the ability to elicit formation of antibodies that

cross react with naturally occurring GnRH. Particularly contemplated herein are repeated sequences of GnRH polypeptides such as in the oligomer depicted in Figure 1B (wherein each of the selected GnRH polypeptides comprises a N-terminal Gln substitution, and further wherein every other GnRH polypeptide comprises an Asp residue substitution at position 2). Epitopes of GnRH are also captured by the definition.

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. Since GnRH is a very small molecule, the identification of epitopes thereof which are able to elicit an antibody response is readily accomplished using techniques well known in the art. See, e.g., Geysen et al. *Proc. Natl. Acad. Sci. USA* (1984) 81:3998-4002 (general method of rapidly synthesizing peptides to determine the location of immunogenic epitopes in a given antigen); U.S. Patent No. 4,708,871 (procedures for identifying and chemically synthesizing epitopes of antigens); and Geysen et al., *Molecular Immunology* (1986) 23:709-715 (technique for identifying peptides with high affinity for a given antibody).

As used herein the term "T-cell epitope" refers to a feature of a peptide structure which is capable of inducing T-cell immunity towards the peptide structure or an associated hapten. In this regard, it is accepted in the art that T-cell epitopes comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules, (Unanue et al., *Science* (1987) 236:551-557). Conversion of polypeptides to MHC class II-associated linear peptide determinants (generally between 5 - 14 amino acids in length) is termed "antigen processing" which is carried out by antigen

presenting cells (APCs). More particularly, a T-cell epitope is defined by local features of a short peptide structure, such as primary amino acid sequence properties involving charge and hydrophobicity, and certain types of secondary structure, such as helicity, that do not depend on the folding of the entire polypeptide. Further, it is believed that short peptides capable of recognition by helper T-cells are generally amphipathic structures comprising a hydrophobic side (for interaction with the MHC molecule) and a hydrophilic side (for interacting with the T-cell receptor), (Margalit et al., *Computer Prediction of T-cell Epitopes*, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116) and further that the amphipathic structures have an α-helical configuration (see, e.g., Spouge et al., *J. Immunol.* (1987) 138:204-212; Berkower et al., *J. Immunol.* (1986) 136:2498-2503).

Hence, segments of proteins which include T-cell epitopes can be readily predicted using numerous computer programs. (See e.g., Margalit et al., *Computer Prediction of T-cell Epitopes*, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116). Such programs generally compare the amino acid sequence of a peptide to sequences known to induce a T-cell response, and search for patterns of amino acids which are believed to be required for a T-cell epitope.

An "immunogenic protein" or "immunogenic amino acid sequence" is a protein or amino acid sequence, respectively, which elicits an immunological response in a subject to which it is administered. Under the invention, a "GnRH immunogen" refers to a GnRH molecule which, when introduced into a host subject, stimulates an immune response. In this regard, a GnRH immunogen includes a multimer

corresponding to more than one selected GnRH polypeptide; and, more particularly, to a multimer having either multiple or tandem repeats of selected GnRH polypeptide sequences, multiple or tandem repeats of selected GnRH epitopes, or any conceivable combination thereof.

An "immunological response" to an antigen or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or γδ T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. An immunological response can be detected using any of several immunoassays well known in the art.

The term "leukotoxin polypeptide" or "LKT polypeptide" intends a polypeptide which includes at least one T-cell epitope and is derived from a protein belonging to the family of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (Highlander et al., *DNA* (1989) 8:15-28), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from *P. haemolytica* and *Actinobacillus pleuropneumoniae*, as well as *E. coli* alpha hemolysin (Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236; Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35; Welch, *Mol. Microbiol.* (1991) 5:521-528). This family of toxins is known as the "RTX" family of toxins (Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35). In addition, the term "leukotoxin polypeptide" refers to a leukotoxin polypeptide which is chemically synthesized, isolated from an organism expressing the same, or recombinantly

produced. Furthermore, the term intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native leukotoxin molecule. Thus, the term includes both full-length and partial sequences, as well as analogues. Although native full-length leukotoxins display cytotoxic activity, the term "leukotoxin" also intends molecules which remain immunogenic yet lack the cytotoxic character of native leukotoxins. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Patent Nos. 4,957,739 and 5,055,400; Lo et al., *Infect. Immun.* (1985) 50:667-67; Lo et al., *Infect. Immun.* (1987) 55:1987-1996; Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236; Highlander et al., *DNA* (1989) 8:15-28; Welch, *Mol. Microbiol.* (1991) 5:521-528. In the chimeras produced according to the present invention, a selected leukotoxin polypeptide sequence imparts enhanced immunogenicity to one or more fused GnRH multimers by providing, among other things, T-cell epitopes comprising small peptide segments in the range of five to fourteen amino acids in length which are capable of complexing with MHC class II molecules for presentation to, and activation of, T-helper cells. As discussed further below, these T-cell epitopes occur throughout the leukotoxin molecule and are thought to be concentrated in the N-terminus portions of leukotoxin, i.e., between amino acid residues 1 to 199.

As used herein, a leukotoxin polypeptide "which lacks cytotoxic activity" refers to a leukotoxin polypeptide as described above which lacks significant cytotoxicity as compared to a native, full-length leukotoxin (such as the full-length *P. haemolytica* leukotoxin described in U.S. Patent Nos.

5,055,400 and 4,957,739) yet still retains immunogenicity and at least one T-cell epitope. Leukotoxin polypeptides can be tested for cytotoxic activity using any of several known assays such as the lactate dehydrogenase release assay, described by Korzeniewski et al., *Journal of Immunological Methods* 64:313-320, wherein cytotoxicity is measured by the release of lactate dehydrogenase from bovine neutrophils. A leukotoxin molecule is identified as cytotoxic if it causes a statistically significant release of lactate dehydrogenase when compared to a control non-cytotoxic molecule.

The provision of LKT-GnRH chimeras comprising leukotoxin polypeptides which lack cytotoxic activity provides several important benefits. Initially, a leukotoxin polypeptide which lacks cytotoxic activity is desirable since the injection of an active toxin into a subject can result in localized cell death (PMNs and macrophages) and, in turn, cause a severe inflammatory response and abscess at the injection site. In this regard, cytotoxic activity resulting in the killing of macrophages may lead to reduced antigen presentation and hence a suboptimal immune response. The removal of the cytotoxic portion as found in the non-cytotoxic LKT polypeptides used in producing the fusion proteins of the invention also results in a truncated LKT gene which is capable of being expressed at much higher levels than full-length LKT. Further, the use of non-cytotoxic LKT polypeptides in the fusions constructed herein which retain sufficient T-cell antigenicity reduces the overall amount of leukotoxin-GnRH antigen which needs to be administered to a host subject to yield a sufficient B-cell response to the selected GnRH polypeptides. Particular examples of immunogenic leukotoxin polypeptides which lack cytotoxic activity

include LKT 352, LKT 111, and LKT 101 which are described in greater detail below.

By "LKT 352" is meant a protein which is derived from the *lktA* gene present in plasmid pAA352 (Figure 2, ATCC Accession No. 68283). The nucleotide sequence and corresponding amino acid sequence of this gene are described in International Publication No. WO91/15237 and are shown in Figure 3. The gene encodes a truncated leukotoxin, having 914 amino acids and an estimated molecular weight of around 99 kDa, which lacks the cytotoxic portion of the molecule. The truncated gene thus produced is expressed at much higher levels than the full-length molecule (more than 40% of total cell protein versus less than 1% of total cell protein for the full-length form) and is more easily purified. The derived LKT 352 is not necessarily physically derived from the sequence present in plasmid pAA352. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of the protein need only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the LKT polypeptide functions to enhance the immunogenicity of antigen with which it is associated yet also lacks cytotoxic activity.

By "LKT 111" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pCB111 (Figure 6, ATCC Accession No. 69748). The nucleotide sequence of this gene and the corresponding amino acid sequence are shown in Figure 7. The gene encodes a shortened version of leukotoxin which was developed from the recombinant leukotoxin gene present in plasmid pAA352 (Figure 2, ATCC Accession No. 68283) by removal of an internal DNA fragment of approximately 1300 bp in length. The

LKT 111 polypeptide has an estimated molecular weight of 52 kDa (as compared to the 99 kDa LKT 352 polypeptide), but retains portions of the LKT 352 N-terminus containing T-cell epitopes which are necessary for sufficient T-cell immunogenicity, and portions of the LKT 352 C-terminus containing convenient restriction sites for use in producing the fusion proteins of the present invention. Under the invention, the LKT 111 leukotoxin peptide is not necessarily physically derived from the sequence present in plasmid pCB111. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of the protein need only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the protein functions to enhance the immunogenicity of antigen with which it is associated and lacks cytotoxicity.

By "LKT 101" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pAA101 (Figure 10, ATCC Accession No. 67883). The predicted amino acid sequence of the *P. haemolytica* leukotoxin produced from the pAA101 construct is depicted in Figure 11. The LKT 101 polypeptide is expressed from a truncated form of the *lktA* gene which contains the 5' end of the gene up to the unique *Pst*1 restriction endonuclease site. The truncated gene was fused to the β-galactosidase gene (lacZ) to facilitate purification of the LKT 101 polypeptide. Under the invention, the LKT 101 leukotoxin peptide is not necessarily physically derived from the sequence present in plasmid pAA101. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of

the protein need only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the protein functions to enhance the immunogenicity of antigen with which it is associated and lacks cytotoxicity.

A leukotoxin-GnRH polypeptide chimera displays "increased immunogenicity" when it possesses a greater capacity to elicit an immune response than the corresponding one or more GnRH multimers alone. Such increased immunogenicity can be determined by administering the particular leukotoxin-GnRH polypeptide and GnRH multimer controls to animals, and comparing anti-GnRH antibody titres thus obtained using standard assays such as radioimmunoassays and ELISAs, well known in the art.

"Recombinant" proteins or polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" proteins or polypeptides are those prepared by chemical synthesis.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* or *in vitro* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

DNA "control sequences" refer collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into mRNA, which is then translated into a chimeric polypeptide encoded by the two coding sequences. The coding sequences need not be contiguous to one another so long as the transcribed sequence is ultimately processed to produce the desired chimeric protein. A control sequence is "operably linked to" a coding sequence when it controls the transcription of the coding sequence.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become

integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra; DNA Cloning, vols I & II, supra; Nucleic Acid Hybridization, supra.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

By "vertebrate subject" is meant any member of the subphylum chordata, including, without limitation, mammals such as rodents, cattle, pigs, sheep, goats, horses and man; domestic animals such as

dogs and cats; birds, including domestic, wild and game birds such as cocks and hens including chickens, turkeys and other gallinaceous birds. The term does not denote a particular age. Thus, both adult and newborn animals are intended to be covered.

B. General Methods

Central to the instant invention is the discovery that leukotoxin polypeptides, when coupled to selected GnRH polypeptide repeats (or multimers), are able to confer superior immunogenicity to the associated GnRH moieties. In this regard, leukotoxin polypeptides act as carrier proteins which present selected GnRH multimers to a subject's immune system in a highly immunogenic form. Thus, chimeric proteins constructed under the invention may be formulated into vaccine compositions which provide enhanced immunogenicity to GnRH polypeptides presented therewith. Fusion of the leukotoxin gene to selected GnRH polypeptides also facilitates purification of the chimeric protein from cells expressing the same.

Accordingly, exemplified herein are leukotoxin chimeras which include leukotoxin fused to more than one GnRH polypeptide. Particular embodiments of the present invention include chimeras comprising a leukotoxin polypeptide fused to one or more GnRH multimers, wherein said multimers have at least one repeating GnRH decapeptide sequence, or at least one repeating unit of a sequence corresponding to at least one epitope of a selected GnRH molecule. Further, the selected GnRH peptide sequences may all be the same, or may correspond to different derivatives, analogues, variants or epitopes of GnRH so long as they retain the ability to elicit an immune response. A representative nucleotide sequence of a GnRH decapeptide is depicted in Figure 1A. The

subject GnRH sequence is modified by the substitution of a glutamine residue at the N-terminal in place of pyroglutamic acid which is found in the native sequence. This particular substitution renders a molecule that retains the native glutamic acid structure but also preserves the uncharged structure of pyroglutamate. Accordingly, the resulting peptide does not require cyclization of the glutamic acid residue and may be produced in the absence of conditions necessary to effect cyclization.

Because the GnRH sequence is relatively short, it can easily be generated using synthetic techniques, as described in detail below. Under the invention, a leukotoxin polypeptide sequence is used to confer immunogenicity upon associated GnRH polypeptides (as a carrier protein) in order to help elicit an adequate immune response toward endogenous GnRH in a vertebrate subject. In this manner, immunization with GnRH can regulate fertility in a vaccinated subject by disruption of estrous cycles or spermatogenesis. A detailed discussion of GnRH can be found in U.S. Patent No. 4,975,420.

It is a particular object of the invention to provide a reliable and effective alternative to invasive sterilization procedures currently practiced in domestic and farm animal husbandry, such as surgical castration, surgical ovariohysterectomy and the like. Immunosuppression of reproductive activity in vertebrate subjects using leukotoxin-GnRH chimeras constructed according to the present invention provides an effective alternative in that the constructs effect uniform inactivation of reproductive activity in immunized animals. In this regard, a suitable sterilization vaccine product must serve to uniformly inactivate reproductive capabilities in individual animals in response to a minimum of

vaccinations in order to provide a successful alternative to surgical procedures. This feature is particularly important for immunosterilization of herd animals, and particularly where it is desired to immunocastrate male piglets to prevent "boar taint" which is produced by the synthesis of sex steroids in normally functioning testicles of male piglets. See e.g. Meloen et al., *Vaccine* (1994) 12(8):741-746. Prior attempts at developing such a product have not produced uniform results due to the insufficient immunogenicity of GnRH peptides and/or related carrier systems, and the resultant inability of various prior GnRH-based vaccines to induce sufficient immune responses toward endogenous GnRH.

It is also a particular object of the invention to provide a method for reducing the incidence of mammary tumors in mammalian subjects using the leukotoxin-GnRH fusion molecules produced herein in a vaccine to block GnRH-regulated ovarian functions such as the production of the ovarian hormones estrogen and progesterone in vaccinated subjects. The role of estrogen and progesterone in the etiology of mammary tumors is well established. These ovarian steroids are important in the early stages of the cancer, but once the mammary tumors become established, some tumors become steroid independent. See e.g., the *Textbook of Endocrinology*, 7th Edition, Wilson et al. (eds), (1985) pp 68-69. Estrogen and progesterone are also known to be carcinogenic and primarily responsible for mammary tumors in dogs.

Accordingly, leukotoxin-GnRH polypeptide chimeras contemplated herein comprise one or more GnRH portions having a plurality of selected GnRH polypeptide sequences in order to render a more immunogenic GnRH peptide antigen. This feature is

based on the recognition that endogenous proteins in general may be rendered effective autoantigens by multimerization of their epitopes as described in detail above. More particularly, the GnRH portions of the present leukotoxin-GnRH chimeras may comprise either multiple or tandem repeats of selected GnRH sequences, multiple or tandem repeats of selected GnRH epitopes, or any conceivable combination thereof. GnRH epitopes may be identified using techniques as described in detail above, or fragments of GnRH proteins may be tested for immunogenicity and active fragments used in compositions in lieu of the entire polypeptide. When more than one GnRH multimers are included in the chimeric molecules, each GnRH portion can be the same or different from other included GnRH portions in the molecule.

The sequence of one particular GnRH portion used herein is depicted in Figure 1B wherein four GnRH sequences, indicated at (1), (2), (3) and (4) respectively, are separated by triplet amino acid spacer sequences comprising various combinations of serine and glycine residues. In the subject oligomer, every other GnRH sequence (those indicated at (2) and (4), respectively) contains a non-conservative amino acid substitution at the second position of the GnRH decapeptide comprising an Asp residue in place of the His residue found in the native GnRH sequence. The alternating GnRH multimeric sequence thus produced renders a highly immunogenic GnRH antigen peptide for use in the fusion proteins of the invention. Other GnRH analogues corresponding to any single or multiple amino acid additions, substitutions and/or deletions are also particularly contemplated herein for use in either repetitive or alternating multimeric sequences. In one particular leukotoxin-GnRH fusion, four copies of the GnRH portion depicted in Figure 1B are fused to

a leukotoxin molecule such that the leukotoxin molecule is flanked on its N- and C- terminus with two copies of the subject GnRH multimer.

Furthermore, the particular GnRH portion depicted in Figure 1B contains spacer sequences between the GnRH moieties. The strategic use of various spacer sequences between selected GnRH polypeptides is used herein to confer increased immunogenicity on the subject constructs. Accordingly, under the invention, a selected spacer sequence may encode a wide variety of moieties of one or more amino acids in length. Selected spacer groups may preferably provide enzyme cleavage sites so that the expressed chimera can be processed by proteolytic enzymes *in vivo* (by APC's or the like) to yield a number of peptides, each of which contain at least one T-cell epitope derived from the carrier portion (leukotoxin portion), and which are preferably fused to a substantially complete GnRH polypeptide sequence. The spacer groups may be constructed so that the junction region between selected GnRH moieties comprises a clearly foreign sequence to the immunized subject, thereby conferring enhanced immunogenicity upon the associated GnRH peptides. Additionally, spacer sequences may be constructed so as to provide T-cell antigenicity, such as those sequences which encode amphipathic and/or α-helical peptide sequences which are generally recognized in the art as providing immunogenic helper T-cell epitopes. The choice of particular T-cell epitopes to be provided by such spacer sequences may vary depending on the particular vertebrate species to be vaccinated. Although particular GnRH portions are exemplified which include spacer sequences, it is also an object of the invention to provide one or more GnRH multimers

comprising directly adjacent GnRH sequences (without intervening spacer sequences).

The leukotoxin-GnRH polypeptide complex can be conveniently produced recombinantly as a chimeric protein. The GnRH portions of the chimera can be fused 5' and/or 3' to the leukotoxin portion of the molecule, one or more GnRH portions may be located at sites internal to the leukotoxin molecule, or the chimera can comprise any combination of GnRH portions at such sites. The nucleotide sequence coding for full-length *P. haemolytica* A1 leukotoxin has been determined. See, e.g., Lo, *Infect. Immun.* (1987) 55:1987-1996; U.S. Patent No. 5,055,400. Additionally, several variant leukotoxin gene sequences are disclosed herein.

Similarly, the coding sequences for porcine, bovine and ovine GnRH have been determined, (Murad et al., *Hormones and Hormone Antagonists*, in The Pharmacological Basis of Therapeutics, Sixth Edition (1980)), and the cDNA for human GnRH has been cloned so that its sequence has been well established (Seeburg et al., *Nature* (1984) 311:666-668). Additional GnRH polypeptides of known sequences have been disclosed, such as the GnRH molecule occurring in salmon and chickens (International Publication No. WO 86/07383, published 18 December 1986). The GnRH coding sequence is highly conserved in vertebrates, particularly in mammals; and porcine, bovine, ovine and human GnRH sequences are identical to one another. The desired leukotoxin and GnRH genes can be cloned, isolated and ligated together using recombinant techniques generally known in the art. See, e.g., Sambrook et al., supra.

Alternatively, DNA sequences encoding the chimeric proteins can be prepared synthetically rather than cloned. The DNA sequence can be designed with

the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature* (1981) 292:756; Nambair et al. *Science* (1984) 223:1299; Jay et al. *J. Biol. Chem.* (1984) 259:6311.

Once coding sequences for the chimeric proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, generally, DNA Cloning: Vols. I & II, supra; T. Maniatis et al., supra; B. Perbal, supra.

The fusion gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the chimeric protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence.

The chimeric proteins of the present invention can be expressed using, for example, native *P. haemolytica* promoter, the *E. coli* tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular fusion coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular chimeric protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above.

Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogues of the chimeric proteins of interest. Mutants or analogues may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The chimeric protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified

directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The chimeric proteins of the present invention may also be produced by chemical synthesis, such as by solid phase peptide synthesis, based on the determined amino acid sequences. Such methods are known to those skilled in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology, supra*, Vol. 1, for classical solution synthesis.

Subjects can be immunized against endogenous GnRH by administration of vaccine compositions which include the present chimeric leukotoxin-GnRH proteins. Prior to immunization, it may be desirable to further increase the immunogenicity of a particular chimeric protein. This can be accomplished in any one of several ways known to those of skill in the art. For example, the leukotoxin-GnRH polypeptide fusion protein may be administered linked to a secondary carrier. For example, a fragment may be conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and

inactive virus particles. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art.

The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or selected GnRH polypeptides) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the chimeric proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651. Also useful is a fusion product of a viral protein and a leukotoxin-GnRH immunogen, where that fusion product is made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the fusion proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

The chimeric proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will

find use herein include, but are not limited to, the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel chimeric proteins can be constructed as follows. The DNA encoding the particular leukotoxin-GnRH chimeric protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant chimeric protein into the viral genome. The resulting TK-recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

It is also possible to immunize a subject with the present chimeric proteins, either administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the ef-

fectiveness of the vaccine. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990. The composition or formulation to be administered will, in any event, contain a quantity of the protein adequate to achieve the desired immunized state in the subject being treated.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 1% to about 30% of the active ingredient, preferably about 2% to about 20%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention.

The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the chimeric proteins into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The chimeric proteins can also be presented using implanted mini-pumps, well known in the art.

Furthermore, the chimeric proteins (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

To immunize a subject, a selected GnRH-leukotoxin chimera is administered parenterally, usually by intramuscular injection in an appropriate vehicle. Other modes of administration, however, such as subcutaneous, intravenous injection and intranasal delivery, are also acceptable. Injectable vaccine

formulations will contain an effective amount of the active ingredient in a vehicle, the exact amount being readily determined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired.

With the present vaccine formulations, approximately 1 μg to 1 mg, more generally 5 μg to 200 μg of GnRH polypeptide per mL of injected solution, should be adequate to raise an immunological response when administered. In this regard, the ratio of GnRH to leukotoxin in the Leukotoxin-GnRH antigens of the subject vaccine formulations will vary based on the particular leukotoxin and GnRH polypeptide moieties selected to construct those molecules. More particularly, in the leukotoxin-GnRH polypeptides used in producing the vaccine formulations under the invention, there will be about 1 to 40% GnRH, preferably about 3 to 30% and most preferably about 7 to 27% GnRH polypeptide per fusion molecule. Increases in the percentage of GnRH present in the LKT-GnRH antigens reduces the amount of total antigen which must be administered to a subject in order to elicit an effective B-cell response to GnRH. Effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the particular leukotoxin-GnRH polypeptide in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

C. Experimental

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See Sambrook et al., supra. Restriction enzymes, T_4 DNA ligase, *E. coli*, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double-stranded DNA fragments were separated on agarose gels.

cDNA and genomic libraries were prepared by standard techniques in pUC13 and the bacteriophage lambda gt11, respectively. See DNA CLONING: Vols I and II, supra.

P. haemolytica biotype A, serotype 1 ("A1") strain B122 was isolated from the lung of a calf which died of pneumonic pasteurellosis and was stored at -70°C in defibrinated blood. Routine propagation was carried out on blood agar plates or in brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5% (v/v) horse serum (Gibco Canada Ltd., Burlington, Canada). All cultures were incubated at 37°C.

Example 1

Isolation of P. haemolytica Leukotoxin Gene

To isolate the leukotoxin gene, gene libraries of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See, Lo et al., *Infect. Immun.*, supra; DNA CLONING: Vols. I and II, supra; and Sambrook et al., supra. A genomic library was constructed in the plasmid vector pUC13 and a DNA library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates with bovine neutrophils and subsequently measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be identical to a leukotoxin gene cloned previously. See, Lo et al., *Infect. Immun.*, supra. To confirm this, smaller fragments were re-cloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned. Progressively larger clones were isolated by carrying out a chromosome walk (5' to 3' direction) in order to isolate full-length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This construct contained the entire leukotoxin gene sequence.

lktA, a *Mae*I restriction endonuclease fragment from pAA114 which contained the entire leukotoxin gene, was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and ligated into the *Sma*I site of the cloning vector pUC13. This plasmid was named pAA179. From this, two expression constructs were made in the ptac-based vector pGH432:lacI digested with *Sma*I. One, pAA342, consisted of the 5'-*Aha*III fragment of the lktA gene while the other, pAA345, contained the entire *Mae*I fragment described above. The clone pAA342 expressed a truncated leukotoxin peptide at high levels while pAA345 expressed full length leukotoxin at very low levels. Therefore, the 3' end of the lktA gene (*Sty*I *Bam*HI fragment from pAA345) was ligated to *Sty*I *Bam*HI-digested pAA342, yielding the plasmid pAA352. The structure of pAA352 is shown in Figure 2 and the nucleotide sequence and predicted amino acid sequence of *P. haemolytica* leukotoxin produced from the pAA352 construct (hereinafter LKT 352) is shown in Figure 3.

Several truncated versions of the leukotoxin gene were expressed from pAA114. These truncated forms were fusions with the B-galactosidase (lacZ) gene. Two fragments, LTX1.1 and LTX3.2, from an *Eco*RV *Pst*1 double digest, were isolated from pAA114 as purified restriction fragments (1.0 kb and 2.1 kb, respectively). These fragments were cloned into the cloning vector pTZ18R that had been digested with *Hinc*II and *Pst*1. The resulting vector, termed pLTX3P.1, was used to transform *E. coli* strain JM105. Transformed cells were identified by plating on media containing ampicillin plus Xgal and IPTG. Blue colonies indicated the presence of a functional lacZ gene. DNA from the transformed cells was analyzed by restriction endonuclease digestion and found to

contain the 5' end of the leukotoxin gene (lktC and lktA).

A leukotoxin *Eco*RV/*Pst*1 5'-fragment (from pLTX3P.1) was subcloned into the cloning vector pBR325 that had been digested with *Eco*R1 and *Pst*1. The pBR325 plasmid also contained the native leukotoxin promoter (obtained from pLTX3P.1) and a promoterless, full length lacZ gene. The resulting construct was used to transform *E. coli* JM105 and blue colonies were isolated from Xgal agar. The new construct was termed pAA101 (ATCC No. 67883) and is depicted in Figure 10. The predicted amino acid sequence of the *P. haemolytica* leukotoxin produced from the pAA101 construct (hereinafter LKT 101) is depicted in Figure 11.

Example 2

Construction of LKT-GnRH Fusions

Representative LKT-GnRH fusions were constructed as follows. Oligonucleotides containing sequences corresponding to single copy GnRH and GnRH as four multiple repeats were constructed on a Pharmacia Gene Assembler using standard phosphoramidite chemistry. The sequences of these oligonucleotides are shown in Figures 1A and 1B. The subject oligonucleotides were annealed and ligated into the vector pAA352 (ATCC No. 68283, and described above), which had been digested with the restriction endonuclease *Bam*H1. This vector contains the *P. haemolytica* leukotoxin gene. The ligated DNA was used to transform *E. coli* strain MH3000. Transformants containing the oligonucleotide inserts were identified by restriction endonuclease mapping.

An eight copy GnRH tandem repeat sequence was prepared by annealing the four copy GnRH oligonucleotides and ligating them into a vector which

had been digested with the restriction endonuclease *Bam*H1. The oligomers were designed to disable the upstream *Bam*H1 site when inserted and to ensure that the insertion of additional copies of the oligomer would be oriented in the proper reading frame. The sequence of the subject oligonucleotide is shown in Figure 1B. Plasmid DNA from the *E. coli* MH3000 strain was then isolated and used to transform the strain JM105. The recombinant plasmids were designated pCB113 (LKT 352:4 copy GnRH, ATCC Accession No. 69749) and pCB112 (LKT 352:8 copy GnRH). Recombinant plasmid pCB113 is shown in Figure 4, plasmid pCB112 is identical to pCB113 except that the multiple copy GnRH sequence (corresponding to the oligomer of Figure 1B) was inserted twice as described above. The nucleotide sequence of the recombinant LKT-GnRH fusion of pCB113 is shown in Figure 5. The nucleotide sequence of the recombinant LKT-GnRH fusion pCB112 is identical except that the multiple copy GnRH sequence was inserted twice.

Example 3

Construction of Shortened LKT Carrier Peptide

A shortened version of the recombinant leukotoxin peptide was constructed from the recombinant gene present on the plasmid pAA352 (as described above). The shortened LKT gene was produced by deleting an internal DNA fragment of approximately 1300 bp in length from the recombinant LKT gene as follows.

The plasmid pCB113, (ATCC Accession No. 69749) which includes the LKT 352 polypeptide fused to four copies of the GnRH polypeptide, was digested with the restriction enzyme *Bst*B1 (New England Biolabs). The resultant linearized plasmid was then digested with mung-bean nuclease (Pharmacia) to remove the

single stranded protruding termini produced by the *Bst*B1 digestion. The blunted DNA was then digested with the restriction enzyme *Nae*1 (New England Biolabs), and the digested DNA was loaded onto a 1% agarose gel where the DNA fragments were separated by electrophoresis. A large DNA fragment of approximately 6190 bp was isolated and purified from the agarose gel using a Gene Clean kit (Bio 101), and the purified fragment was allowed to ligate to itself using bacteriophage T4 DNA ligase (Pharmacia). The resulting ligation mix was used to transform competent *E. coli* JM105 cells, and positive clones were identified by their ability to produce an aggregate protein having a molecular weight of approximately 57 KDa. The recombinant plasmid thus formed was designated pCB111, (ATCC Accession No. 69748), and produces a shortened leukotoxin polypeptide (hereinafter referred to as LKT 111) fused to four copies of GnRH polypeptide. The structure of pCB111 is shown in Figure 6. Plasmid pCB114 is identical to pCB111 except that the multiple copy GnRH sequence (corresponding to the oligomer of Figure 1B) was inserted twice. The nucleotide sequence of the recombinant LKT-GnRH fusion of pCB111 is shown in Figure 7, the nucleotide sequence of the recombinant LKT-GnRH fusion of pCB114 is identical except that the multiple copy GnRH sequence was inserted twice.

The nucleotide sequence of the ligation fusion point of the subject clones has been confirmed by sequencing with a bacteriophage T7 polymerase sequencing kit (Pharmacia). The nucleotide sequences of these fusion points are shown in Figure 8.

Example 4

Construction of an LKT-GnRH Fusion Having 8 Copy Amino Terminal and Carboxyl Terminal GnRH Multimers

A recombinant LKT-GnRH fusion molecule having two 8 copy GnRH multimers, one arranged at the N'-terminus of LKT 111 and the other arranged at the C'-terminus of LKT 111, was constructed from the LKT-GnRH fusion sequence obtained from the pCB114 plasmid by ligating the multiple copy GnRH sequence (corresponding to the oligomer of Figure 1B) twice at the 5' end of the LKT 111 coding sequence. A synthetic nucleic acid molecule having the following nucleotide sequence: 5'-ATGGCTACTGTTATAGATCGATCT-3' was ligated at the 5' end of the multiple copy GnRH sequences. The synthetic nucleic acid molecule encodes an eight amino acid sequence (Met-Ala-Thr-Val-Ile-Asp-Arg-Ser). The resulting recombinant molecule thus contains in the order given in the 5' to 3' direction: the synthetic nucleic acid molecule; a nucleotide sequence encoding a first 8 copy GnRH multimer; a nucleotide sequence encoding the shortened LKT peptide (LKT 111); and a nucleotide sequence encoding a second 8 copy GnRH multimer.

The recombinant molecule was circularized, and the resulting molecule was used to transform competent *E. coli* JM105 cells. Positive clones were identified by their ability to produce an aggregate protein having a molecular weight of approximately 74 KDa. The recombinant plasmid thus formed was designated pCB122 which produces the LKT 111 polypeptide fused to 16 copies of GnRH polypeptide. The nucleotide sequence of the recombinant LKT-GnRH fusion of pCB122 is shown in Figures 9-1 through 9-6.

Example 5

Purification of LKT-antigen Fusions

The recombinant LKT-GnRH fusions from Examples 2, 3 and 4 were purified using the following procedure. For each fusion, five to ten colonies of the transformed *E. coli* strains were inoculated into 10 mL of TB broth supplemented with 100 micrograms/mL of ampicillin and incubated at 37°C for 6 hours on a G10 shaker, 220 rpm. Four mL of this culture was diluted into each of two baffled Fernbach flasks containing 400 mL of TB broth + ampicillin and incubated overnight as described above. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in polypropylene bottles, 500 mL volume, using a Sorvall GS3 rotor. The pellet was resuspended in an equal volume of TB broth containing ampicillin which had been prewarmed to 37°C (i.e., 2 x 400 ml), and the cells were incubated for 2 hours as described above.

3.2 mL of isopropyl-B,D-thiogalactopyranoside (IPTG, Gibco/BRL), 500 mM in water (final concentration = 4 mM), was added to each culture in order to induce synthesis of the recombinant fusion proteins. Cultures were incubated for two hours. Cells were harvested by centrifugation as described above, resuspended in 30 mL of 50 mM Tris-hydrochloride, 25% (w/v) sucrose, pH 8.0, and frozen at -70°C. The frozen cells were thawed at room temperature after 60 minutes at -70°C, and 5 mL of lysozyme (Sigma, 20 mg/mL in 250 mM Tris-HCl, pH 8.0) was added. The mixture was vortexed at high speed for 10 seconds and then placed on ice for 15 minutes. The cells were then added to 500 mL of lysis buffer in a 1000 mL beaker and mixed by stirring with a 2 mL pipette. The beaker containing the lysed cell suspension was placed on ice and sonicated for a total of 2.5 minutes (5-30 second

bursts with 1 minute cooling between each) with a Braun sonicator, large probe, set at 100 watts power. Equal volumes of the solution were placed in Teflon SS34 centrifuge tubes and centrifuged for 20 minutes at 10,000 rpm in a Sorvall SS34 rotor. The pellets were resuspended in a total of 100 mL of sterile double distilled water by vortexing at high speed, and the centrifugation step repeated. Supernatants were discarded and the pellets combined in 20 mL of 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (Tris-buffered saline) and the suspension frozen overnight at -20°C.

The recombinant suspension was thawed at room temperature and added to 100 mL of 8 M Guanidine HCl (Sigma) in Tris-buffered saline and mixed vigorously. A magnetic stir bar was placed in the bottle and the solubilized sample was mixed at room temperature for 30 minutes. The solution was transferred to a 2000 mL Erlenmeyer flask and 1200 mL of Tris-buffered saline was added quickly. This mixture was stirred at room temperature for an additional 2 hours. 500 mL aliquots were placed in dialysis bags (Spectrum, 63.7 mm diameter, 6,000-8,000 MW cutoff, #132670, from Fisher scientific) and these were placed in 4,000 mL beakers containing 3,500 mL of Tris-buffered saline + 0.5 M Guanidine HCl. The beakers were placed in a 4°C room on a magnetic stirrer overnight after which dialysis buffer was replaced with Tris-buffered saline + 0.1 M Guanidine HCl and dialysis continued for 12 hours. The buffer was then replaced with Tris-buffered saline + 0.05 M Guanidine HCl and dialysis continued overnight. The buffer was replaced with Tris-buffered saline (no guanidine), and dialysis continued for 12 hours. This was repeated three more times. The final solution was poured into a 2000 mL plastic roller bottle (Corning) and 13 mL of 100 mM PMSF (in

ethanol) was added to inhibit protease activity. The solution was stored at -20°C in 100 mL aliquots.

To confirm that the fusion proteins had been isolated, aliquots of each preparation were diluted 20-fold in double distilled water, mixed with an equal volume of SDS-PAGE sample buffer, placed in a boiling water bath for five minutes and run through 12% polyacrylamide gels. Recombinant leukotoxin controls were also run.

All fusion proteins were expressed at high levels as inclusion bodies. The predicted molecular weights based on the DNA sequences of the fusion proteins were 104,869 (LKT 352::4 copy GnRH, from pCB113); 110,392 (LKT 352::8 copy GnRH, from pCB112); 57,542 (LKT 111::4 copy GnRH, from pCB111); 63,241 (LKT 111::8 copy GnRH from pCB114); and 73,886 (8 copy GnRH::LKT 111::8 copy GnRH from pCB122). The predicted molecular weight of the recombinant LKT 352 molecule was 99,338, and the predicted molecular weight of the recombinant LKT 111 molecule was 51,843.

Example 6

In Vivo Immunologic Activity of LKT-GnRH Fusions

To test for the ability of LKT-GnRH fusions to induce an anti-GnRH immunological response *in vivo*, and to compare this response to other GnRH carrier conjugates, the following vaccination trial was performed. Three groups of 8 male pigs, approximately 8 weeks of age (35-50 kg) were used which were Specific Pathogen Free. The animals were maintained in a minimal disease facility and were vaccinated on days 0 and 21 of the trial with the following formulations:

Group 1 -- placebo which consisted of saline formulated in Emulsigen Plus adjuvant containing 15 mg of dimethyldioctadecylammonium bromide (DDA) (2 ml);

Group 2 -- LKT 352-GnRH (250 μg LKT, prepared as described in the previous examples) formulated in the same adjuvant (2 ml);

Group 3 -- VP6-GnRH, 0.5 μg VP6 and 5 μg GnRH, formulated in the same adjuvant (2 ml). The VP6 preparation was made as described in U.S. Patent No. 5,071,651, using the binding peptide described therein.

Blood samples were taken on days 0, 21 and 35, allowed to clot, centrifuged at 1500 g, and the serum removed. The serum antibody titres against GnRH were measured using the RIA procedure of Silversides et al., *J. Reprod. Immunol.* (1985) 7:171-184.

The results of this trial indicated that only those animals immunized with the LKT 352-GnRH formulation produced significant titres against GnRH (titres >1:70). Neither the placebo nor the VP6-GnRH groups produced anti-GnRH titres. Previously, multiple vaccinations with doses of GnRH of more than 100 μg, conjugated to other carrier proteins, were required to induce anti-hormone titres. These results indicate that the LKT-GnRH carrier system provides a greatly improved immunogen over prior carrier systems.

Example 7

In Vivo Immunologic Effect of Multiple Tandem GnRH Repeats Ligated to LKT

To test for the ability of recombinant LKT-GnRH fusion proteins containing multiple GnRH polypeptide repeats to induce an anti-GnRH immunological response *in vivo*, the following vaccination trial was performed. Cultures of *E. coli* containing plasmids pCB113 and pCB175 (having 4 and 8 copies of GnRH ligated to LKT 352, respectively) and a plasmid having 1 copy of GnRH ligated to LKT 352 were prepared as described above. Vaccines from each of

the above cultures were formulated to contain the equivalent of 5 μg of GnRH in 0.2 mL of Emulsigen Plus. Three groups of 10 female mice were given two subcutaneous injections 23 days apart and blood samples were collected at days 23, 35 and 44 after the primary injection. Serum antibody titres against GnRH were measured at final dilutions of 1:100 and 1:1000 using a standard radioimmunoassay procedure. If less than 5% of the iodinated GnRH was bound, antibody was deemed to be undetectable. The antibody titres thus obtained are summarized in the Table 1.

The results of this study indicate that equal doses of GnRH presented as multiple tandem repeats (four or eight copy GnRH) gave a dramatic improvement in antibody production over single copy GnRH (as measured by binding to iodinated native GnRH). Further, the above results indicate that a fusion protein comprising a four copy GnRH tandem repeat ligated to LKT 352 represents an effective immunogenic GnRH antigen form, although immunogenicity may be influenced by dose or subject species.

Sample Day	Group 1				Group 2				Group 3			
	LKT 352::1 Copy GnRH				LKT 352::4 Copy GnRH				LKT 352::8 Copy GnRH			
	No. responding		mean response (%)*		No. responding		mean response (%)*		No. responding		mean response (%)*	
	1:100	1:1000	1:100	1:1000	1:100	1:1000	1:100	1:1000	1:100	1:1000	1:100	1:1000
23	0	0	-	-	3	1	16	9	2	0	33	-
35	2	2	45	20	9	9	75	30	7	5	48	41
44	2	2	60	39	10	10	55	43	8	7	57	46

*mean response is the average binding of I^{125}-GnRH of only those animals with binding in excess of 5%.

Table 1

Example 8

In Vivo Immunologic Activity and Biologic Effect of LKT 352::GnRH and LKT 111::GnRH Fusions

To test the ability of fusion proteins comprising multiple tandem repeats of GnRH (ligated to either LKT 352 or LKT 111) to elicit an anti-GnRH immunological response *in vivo* and to manifest a biologic effect *in vivo*, the following vaccination trial was preformed. Cultures of *E. coli* containing plasmids pCB113 and pCB111 (4 copy GnRH ligated to LKT 352 or LKT 111, respectively) were prepared as described above. Vaccines from each of the above cultures were formulated to contain the equivalent of 5 μg of GnRH in 0.2 mL of VSA-3 adjuvant, (a modified Emulsigen Plus adjuvant), with a control vaccine comprising 0.2 mL of the adjuvant also being prepared. Three groups of 5 male Swiss mice were given two subcutaneous injections 21 days apart, with the initial injections (day 0) given at 5-6 weeks of age. On day 49 the subjects were sacrificed.

Immunological activity of the subject GnRH-LKT fusions was assayed by measuring anti-GnRH antibody titres using a standard radioimmunoassay procedure at a 1:1000 serum dilution. Biological effect of the GnRH-LKT fusions was quantified by standard radioimmunoassay of serum testosterone levels with a sensitivity of 25 pg/ml, and testicular tissue was weighed and histologically examined. The results of this trial are summarized in Table 2.

In the trial, all animal subjects injected with GnRH:LKT antigens had readily detectable antibody levels; however, the LKT 111::GnRH fusion (from plasmid pCB111) showed superior immunogenicity as indicated by uniformity of response and titre. Serum testosterone (produced by the testicular Leydig cells) is secreted in a pulsatile manner, and accordingly,

low values and extreme variability of serum levels are expected in normal animal subjects. Under the trial, the control group (receiving the 0.2 mL adjuvant vaccine injections) had normal serum testosterone levels, while both groups of treated subjects had essentially undetectable serum testosterone.

Further under the trial, histological evaluation of testicular tissue revealed varying degrees of Leydig cell atrophy, reduced seminiferous tubule diameter and interruption of spermatogenesis in treated subjects; however, testicular weight remained close to normal in treated animals –even in the presence of high anti-GnRH antibody titres– although there was clear evidence of testicular regression in 2 of 5 subjects receiving the LKT 111::4 copy GnRH fusions.

Accordingly, these results show that multiple copies of GnRH ligated to either LKT 352 or LKT 111 comprise potent immunogens; and further, it is indicated that vaccination with the subject fusion proteins triggers production of antibodies which are able to neutralize endogenous GnRH *in vivo*, and that a concomitant *in vivo* biological effect is discernable in animal subjects receiving such vaccinations.

Animal	Group 1 Control			Group 2 5 μg LKT 352::4 Copy GnRH			Group 3 5 μg LKT 111::4 Copy GnRH		
	Anti-body Titre*	Testicular Wt.(mg)	Serum Testosterone†	Anti-body Titre*	Testicular Wt.(mg)	Serum Testosterone†	Anti-body Titre*	Testicular Wt.(mg)	Serum Testosterone†
1	7.0	252	.04	73.0	282	.13	75.0	163	.00
2	4.0	327	.18	14.0	334	.10	59.0	296	.07
3	0.0	276	2.73	18.0	254	.03	54.0	260	.24
4	0.0	220	.36	55.0	222	.05	66.0	265	.03
5	1.0	232	1.44	61.0	226	.19	64.0	50	.00
Mean	2.4	261	.95	44	263	.10	64	206	.07
Std Error	1.4	19	.51	12	21	.03	4	45	.04

* % Binding of I^{125}-GnRH at a 1:1000 serum dilution
† ng/ml

Table 2

Example 9

In Vivo Immunologic Activity of LKT::GnRH Fusions in Porcine Subjects

To test the ability of fusion proteins comprising multiple tandem repeats of GnRH (ligated to either LKT 352 or LKT 111) to elicit anti-GnRH immunological response *in vivo* in porcine subjects, the following vaccination trial was preformed. Cultures of *E. coli* containing plasmids pCB113, pCB111, pCB175 and pCB114 (LKT 352::4 copy GnRH, LKT 111::4 copy GnRH, LKT 352::8 copy GnRH, and LKT 111::8 copy GnRH, respectively) were prepared as described above. Vaccines from each of the above cultures were formulated to contain the equivalent of 50 μg GnRH and were administered in VSA-3 adjuvant in a 2.0 mL volume. Four groups of 5 male and 5 female weanling pigs, 35 days old (at day 0), were injected at day 0 and reinjected at day 21 of the trial. Blood samples were collected at days 0, 21 and 35, with anti-GnRH antibody titres being measured at a final dilution of 1:1000 using a standard radioimmunoassay procedure. The assay results are summarized in Table 3.

Under the trial, anti-GnRH antibodies could not be detected in any subjects prior to immunization, but were readily detected in most subjects by day 35 (one subject in treatment group 4 died due to an infection unrelated to treatment). The results in this trial indicate that fusion proteins comprising multiple GnRH repeats ligated to either a LKT 352 or LKT 111 carrier polypeptide form useful immunogens in porcine subjects. Based on the predicted molecular weights of the decapeptide GnRH (1,200), the LKT 111 polypeptide (52,000) and the LKT 352 polypeptide (100,000), the percentages of GnRH in the LKT-GnRH antigen fusions are as follows: 4.9% (LKT 352::4 copy GnRH); 8.5% (LKT 111::4 copy GnRH); 9.3% (LKT 352::8

copy GnRH) and 15.7% (LKT 111::8 copy GnRH). Accordingly, the practical result thus obtained indicates that by using LKT-GnRH fusions comprising the LKT 111 polypeptide carrier, the overall amount of antigen (LKT-GnRH) administered to the subject may be halved (as compared to vaccination compositions using the LKT 352 carrier polypeptide system) to obtain an equivalent anti-GnRH response.

Animal Number	Group 1	Group 2	Group 3	Group 4
	LKT 352::4 copy GnRH 50 µg	LKT 111::4 copy GnRH 50 µg	LKT 352::8 copy GnRH 50 µg	LKT 111:: 8 copy GnRH 50 µg
	day 35 1:1000 dilution	day 35 1:1000 dilution	day 35 1:1000 dilution	day 35 1:1000 dilution
1	♂ 47.7	♀ 46.0	♂ 68.3	♂ 51.0
2	♀ 50.3	♂ 71.6	♂ 65.1	♂ 31.7
3	♀ 66.0	♀ 21.4	♀ 50.7	♀ 35.7
4	♀ 70.2	♂ 46.2	♂ 4.7	♀ 65.9
5	♂ 17.3	♀ 48.9	♀ 38.3	♀
6	♂ 18.3	♂ 69.4	♀ 17.4	♂ 11.3
7	♀ 14.7	♂ 47.9	♀ 51.4	♀ 28.3
8	♂ 37.0	♀ 44.4	♂ 18.0	♂ 43.0
9	♂ 26.0	♂ 70.8	♂ 83.5	♀ 78.7
10	♀ 2.7	♀ 37.8	♀ 24.2	♂ 55.9
Mean	35.0	50.4	42.2	44.6
Standard Deviation	7.3	5.1	8.1	6.9
Responders	9/10	10/10	9/10	9/9

Table 3

Example 10

Evaluation of LKT 111::8 Copy GnRH Immunocastration Vaccine Efficiency

To evaluate the efficacy and commercial usefulness of a vaccine formulation containing the LKT 111::8 copy GnRH fusion protein, the following vaccination trial was carried out. A culture of *E. coli* containing the plasmid pCB114 (LKT 111::8 copy GnRH) was prepared as described above. A vaccine formulation from the above culture was prepared which contained the equivalent of 50 μg GnRH. The vaccine formulation was administered in VSA-3 adjuvant at a 2.0 mL final volume. Three treatment groups, with 30 male pigs (boars) each, were established. The three groups consisted of 30 barrows (boars surgically castrated before sexual maturity), 30 control boars and 30 immunocastrates (boars castrated by vaccination with the GnRH immunogen). At weaning (day 21), the barrow and control boar group animals were injected with placebo (VSA-3 adjuvant alone), while the immunocastrate group was injected with the above-described vaccine formulation. When the animals reached a predetermined weight about 3 weeks before slaughter, the immunocastrate group was given a booster dose of the vaccine, while the barrow and control boar groups were again given placebo injections. Measurements included serum antibody titres to GnRH, blood testosterone levels, carcass traits, animal behavior, feed efficiency, rate of weight gain, and salivary gland and body fat androstenone levels (as a measure of boar taint).

(a) Serum Anti-GnRH Antibody Titre:

Immunological activity of the 8 copy GnRH-LKT fusion vaccine formulation was assayed by measuring anti-GnRH antibody titres using a standard

radioimmunoassay procedure at a 1:5000 serum dilution. A comparison of serum antibody titres in the three experimental groups is provided in Figure 12. As can be seen, anti-GnRH antibody titres increased dramatically in the immunocastrate (vaccinated) boars and remained at levels significantly in excess of the minimal amount required to produce a biological effect (approximately 10 to 20 % binding in Figure 12) for over 20 days post vaccination.

(b) Biological Effect of the Immunocastrate Vaccine on Sexual Gland Size:

The biological effect of the 8 copy GnRH-LKT fusion vaccine formulation was determined by comparing the weight and measurements of sexual glands from the control boars and the immunocastrate (vaccinated) boars, as well as by assaying and comparing serum testosterone levels in those two experimental groups. In particular, the bulbourethral glands and testes from the animals were weighed and measured. The results are depicted below in Table 4. As can be seen, the average weight of the bulbourethral glands in the vaccinated animals was reduced approximately 32% relative to the control animals. In addition, the average weight of the testes in the vaccinated animals was reduced approximately 25% relative to the control animals. These results are consistent with reduced testosterone production from the testes in the vaccinated animals.

TABLE 4

Treatment	No. of Animals	Bulbourethral Gland				Testes	
		Average Weight (gm)	% of Control	Average Length (cm)	% of Control	Average Weight (gm)	% of Control
Control Boars	22	60.5±3.5*		11.4±.21		263±10.9	
Immunocastrate Boars	27	41.3±5.2	68.3	9.5±.47	83.3	198±11.3	75.3

*means ± standard errors

The average serum testosterone levels in all three experimental groups was determined using a standard radioimmunoassay of serum testosterone levels with a sensitivity of 25 pg/mL. The assays were conducted on Day 0, Day 7, Day 14, and Day 21 after the booster immunizations (and placebo vaccinations in the control boar and barrow groups). The results of the assays are depicted in Figure 13. As can be seen, the serum testosterone levels in the vaccinated animals decreased after vaccination, while the levels in the control boars increased.

(c) Carcass Composition:

Commercial aspects of the carcass composition of animals from each experimental group were assessed after slaughter of the animals. In particular, average body weights and fat content were determined, average measurements of the loin eye were taken, and the average weight of trimmed hams and loin was determined. The results of the carcass assessments are reported in Table 5. As can be seen, the carcass data show that the control boars and immunocastrates (vaccinated animals) had very similar carcass compositions, whereas the barrows had appreciably more body fat, less body lean. In addition, the growth performance of the barrows reached a plateau over the last 24 days of life (results not shown). These carcass data are consistent with the objective of having the carcass compositions of the immunocastrated animals mimic that of the control boars for all but the final few days of their growing period.

TABLE 5			
Carcass Data			
	Borrows	Control Boars	Immunocastrates
Kill wt (kg)	110.5	115.2	115.4
Fat (mm)	19.1	15.7	15.3
Loin eye (cm^2)	41.5	44.5	44.2
Trim Primal (kg)	27.3	28.4	28.2
Trimmed ham (kg)	7.70	8.23	8.11
Trimmed loin (kg)	7.38	7.79	7.65

(d) Feed Conversion:

The feed conversion efficiency of animals from each of the experimental groups was measured over the period of weaning to slaughter. In particular, average feed conversion efficiency was expressed as the ratio of Kg feed:Kg weight gain in the animals. The results are depicted in Figure 14. As can be seen, feed conversion in the control boars and the immunocastrates (vaccinated animals) was about 10% more efficient than feed conversion in the barrows.

(e) Boar Taint Component Levels:

The ability of the 8 copy GnRH-LKT fusion vaccine formulation to reduce boar taint in vaccinated animals was assessed by assaying the androstenone levels (a boar taint component) in fat and salivary glands of animals from each of the experimental groups. Androstenone levels were quantified by a standard chemical method on fat and salivary gland specimens obtained from each group. The results are reported in Table 6. As can be seen, the control

boars had appreciably higher androstenone concentrations relative to the barrows and the immunocastrates (vaccinated animals).

TABLE 6

	Barrows	Control Boars	Immunocastrates
Fat Androstenone	0.14 μg/g	0.44 μg/g	0.26 μg/g*
Salivary Androstenone	33.76 μg/g	40.46 μg/g	30.18 μg/g

*p less than .01

All of the above results indicate that immunocastration vaccine formulations containing the short LKT::8 copy GnRH fusion molecules provide a commercially viable alternative to surgical castration methods.

Example 11

Comparison of *In Vivo* Immunogenic Activity of Fusion Molecules Having One or Two GnRH Multimers

In order to compare the ability of LKT-GnRH fusion proteins comprising either a single GnRH multimer (containing 8 tandem repeats of GnRH), or two GnRH multimers (both containing 8 tandem repeats of GnRH), to elicit an anti-GnRH immunological response *in vivo*, several vaccination trials were carried out.

Cultures of *E. coli* containing plasmids pCB114 (one 8 copy GnRH multimer, ligated to the C'-terminus of LKT 111), and pCB122 (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. Vaccines derived from cultures containing the pCB114 plasmid were formulated to contain 160 μg of the fusion molecules (25 μg total of GnRH) in a 2 mL final volume of VSA-3

adjuvant. Vaccines derived from cultures containing the pCB122 plasmid were formulated to contain 185 μg of the fusion molecules (50 μg total of GnRH) in a 2 mL final volume of VSA-3 adjuvant. In this manner, the amount of the LKT carrier molecule was kept constant (135 μg total of LKT per formulation) in both preparations. The vaccine formulations were used in the following vaccination trials.

(a) Anti-GnRH Antibody Titre and Functional Activity of the Anti-GnRH Antibody Molecules:

A comparison between anti-GnRH antibody titres elicited by the two experimental vaccine formulations was carried out, wherein the ability of the elicited antibodies to block the effect of endogenously produced GnRH was also assessed. In particular, three groups of male pigs were established as follows: 50 animals were injected with the single GnRH multimer vaccine composition (LKT 111::8 copy GnRH fusions obtained from pCB114), 10 animals were injected with the plural GnRH multimer vaccine composition (8 copy GnRH::LKT 111::8 copy GnRH fusions obtained from pCB122), and 10 control animals were injected with 2 mL of the VSA-3 adjuvant alone.

Vaccinations were carried out at weaning (21 days of age), and the animals were boosted 30 days later. Blood was collected 14 and 28 days after the booster immunization. Serum was obtained and assayed for anti-GnRH antibody titer and serum levels of Luteinizing Hormone (LH). Serum anti-GnRH antibody titres were determined at a final serum dilution of 1:5000 using iodinated GnRH in a standard radioimmunoassay. Serum levels of LH were assayed using porcine LH as a reference standard in a standard radioimmunoassay. The results of the assays, given as mean values ± standard errors, are reported in Table

7. As can be seen by the data depicted in Table 7, anti-GnRH antibody titres were higher in animals injected with the plural GnRH multimer vaccine composition (8 copy GnRH::LKT 111::8 copy GnRH) than seen with the animals receiving the single GnRH multimer vaccine (LKT 111::8 copy GnRH). Further, the animals receiving the plural GnRH multimer vaccine had lower serum LH levels. This reduction in serum LH reflects the ability of the anti-GnRH antibodies produced in the immunized animals to block the effect of endogenously produced GnRH. Finally, 100% of the animals receiving the plural GnRH multimer vaccine responded to the vaccine by producing anti-GnRH antibodies, whereas 90-92% of the animals receiving the single GnRH multimers responded.

TABLE 7

	GnRH Antibodies at Day		Serum LH at Day
Day after the Booster	14	28	14
Treatments 1 (Control)	0.5 ± .3	0.5 ± .3	1.16 ± .22
Treatment 2 LKT III::8 copy GnRH 160 μg (25 μg GnRH)	44.6 ± 4.1	37.2 ± 4.1	0.13 ± .04
Treatment 3 8 copy GnRH::LKT III::8 copy GnRH 185 μg (50 μg GnRH)	60.5 ± 6.9	51.8 ± 7.5	.06 ± .02

(b) Comparison of Anti-GnRH Titres and Assessment of the Effect of Increased Vaccine Dosages:

The immunogenicity of the two vaccine formulations (the 8 copy GnRH single multimer antigen and the 16 copy GnRH plural multimer antigen) was again assessed as follows. Two experimental groups of

20 male pigs each were established. Animals in the first group were vaccinated at weaning (Day 21 of age) with 160 μg of the single multimer antigen preparation, and then boosted 33 days later with the same dosage. Animals in the second group were vaccinated at weaning (Day 21 of age) with 185 μg of the plural multimer antigen preparation and also boosted 33 days later. Blood was collected at 8, 14, and 24 days after the booster injections, and serum was assayed for anti-GnRH antibody molecules at a final dilution of 1:5000 using standard radioimmunoassay as previously described. The results are depicted in Figure 15. As can be seen, the antibody response to the plural multimer vaccine (8 copy GnRH::LKT 111::8 copy GnRH) was higher ($P<.001$) than for the single multimer vaccine (LKT 111::8 copy GnRH). Referring still to Figure 15, the horizontal line at 20% on the Y axis represents an antibody titre which, in previous trials not reported herein, have been shown to suppress secretion of LH in vaccinated animals. Once again, 100% of the animals receiving the plural GnRH multimer vaccine responded (produced anti-GnRH antibodies), while approximately 90-92% of the animals receiving the single multimer vaccine responded.

In order to determine if the increased immunogenicity observed with the plural GnRH multimer vaccine is due to the increased dosage of the GnRH antigen (e.g., 50 μg GnRH in the [8 copy GnRH::LKT 111::8 copy GnRH] vaccine, as compared to 25 μg GnRH in the [LKT 111::8 copy GnRH] vaccine), the following study was carried out. Three groups of 20 pigs each were vaccinated at weaning (21 days of age) and boosted approximately 30 days later with the single GnRH multimer vaccine composition (LKT 111::8 copy GnRH fusions obtained from pCB114) at the following

dosages: 50 μg, 150 μg and 450 μg of the fusion protein, respectively. Blood was collected at 14, 28 and 64 days after the booster injection. Serum was assayed for anti-GnRH antibodies at a final dilution of 1:5000 as described above. The results are reported in Table 8. As can be seen, no appreciable increase in anti-GnRH antibody titres were obtained in response to vaccination with increased dosages of the single GnRH multimer vaccine composition. This indicates that the increased immunogenicity observed with plural GnRH multimer vaccine (8 copy GnRH::LKT 111::8 copy GnRH fusions obtained from pCB122) is not due to increased GnRH antigen concentration; rather the increased immunogenicity is likely due to the three dimensional structure of the particular LKT-GnRH fusion molecule, or in the physical presentation of the GnRH antigen to antibody producing cells.

TABLE 8

Dose (μg) LKT III::8 copy GnRH	% Binding at 1:5000 Dilution at Day after Boost		
	Day 14	Day 28	Day 64
50 μg	60.9 ± 4.8	50.7 ± 5.8	22.0 ± 4.7
150 μg	59.0 ± 4.9	46.0 ± 4.9	16.8 ± 3.6
450 μg	62.6 ± 4.0	56.5 ± 4.7	22.8 ± 4.8

Example 12

Dose Response Study With LKT-GnRH Fusion Molecules Having Two GnRH Multimers

In order to determine optimal dosages of vaccine compositions formed from LKT-GnRH fusion proteins comprising two GnRH multimers (both containing 8 tandem repeats of GnRH), the following *in vivo* dose response study was carried out.

Cultures of *E. coli* containing plasmid pCB122 (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. Seven vaccines derived from cultures containing the pCB122 plasmid were formulated at the following dosages of total fusion protein: 0 μg (control); 1 μg; 5 μg; 10 μg; 20 μg; 40 μg; and 80 μg, each in a 1 mL final volume of VSA-3 adjuvant.

Seven experimental groups of 20 animals each were assembled and vaccinated with the above-described vaccine formulations. A blood sample was taken at day 35 after the vaccination, and anti-GnRH antibody titres were measured at a final dilution of 1:100 in a standard radioimmunoassay as described above. The results of the assay are reported in Table 9. The titres are expressed as % binding as above. As can be seen, statistically 0 μg of the fusion protein was different from all other values. The 1 μg fusion protein dose was lower ($p < .009$) than all other values obtained from groups receiving the protein antigen. The 5 μg dose was less than the 20 μg dose ($p < .06$), however, all values for doses above 10 μg total fusion protein were statistically similar. These data show that the optimal dosage of the vaccine derived from the fusion protein of plasmid pCB122 (8 copy GnRH::LKT 111::8 copy GnRH) is approximately 20 - 40 μg of the fusion protein.

TABLE 9

	8 copy GnRH::LKT 111::8 copy GnRH Dose (μg)						
	0	1	5	10	20	40	80
Titre $\bar{x}$	2.6	20.5	47.9	52.0	59.6	62.0	64.6
$S\bar{x}$	±.6	5.0	5.8	4.6	4.4	3.4	3.6

Example 13

GnRH Immunization of Female Rats

In order to assess biological effects of GnRH immunization of female subjects, the following study was carried out.

Cultures of *E. coli* containing the pCB122 plasmid (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. A vaccine derived from the cultures was formulated to contain 185 μg of the fusion molecules (50 μg total of GnRH) in a 1 mL final volume of VSA-3 adjuvant. The formulation was then used in the following vaccination trial to assess the effect of GnRH immunization on ovarian weight, uterine weight, and serum estrogen concentration in female subjects.

Two experimental groups of female Sprague Dawley rats, 10 animals per group, were assembled. A control group (Group 1) was given a placebo injection (VSA-3 adjuvant only) at day 0 of the trial. Animals in the second group received a single injection of the GnRH/LKT vaccine formulation. Anti-GnRH antibody titres were monitored after treatment, and animals in Group 2 showed a rise in titer that began 21 days after injection to reach maximum levels at approximately day 50 of the study, after which the levels declined gradually until the animals were sacrificed on day 224 of the study.

Ovarian weight, uterine weight, and serum estradiol levels were then determined and recorded. The results of these measurements are depicted in Figure 16. As can be seen, ovarian weights in the treated animals (immunized with the GnRH-LKT vaccine formulation) were reduced dramatically relative to the control animals. Histological examination of the tissue revealed no active follicles in the ovarian

tissue. Uterine weights were also dramatically reduced in the treated animals. Uterine weight provides a good reflection of serum estrogen concentrations, and is related to gonadal steroid secretion. Furthermore, serum estradiol levels were reduced in the treated animals to about 20 pg/mL, whereas serum estradiol was about 50 pg/mL in the control animals. Since estrogen is derived from the ovary, it was expected that the serum estradiol would be reduced in the treated animals. These results demonstrate that the GnRH/LKT immunizations of the present invention are effective in controlling ovarian function, indicating a viable alternative to procedures such as ovariectomy or treatment with GnRH antagonists.

Example 14

Immunocastration of Male Porcine Subjects Using LKT-GnRH Fusion Molecules Having Two GnRH Multimers

In order to determine the ability of vaccine compositions formed from LKT-GnRH fusion proteins having two GnRH multimers (both containing 8 tandem repeats of GnRH) to reduce androstenone in fat, the following study was carried out.

Cultures of *E. coli* containing plasmid pCB122 (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. Vaccine compositions derived from the cultures were prepared as also described above. Four experimental groups of male porcine subjects were formed as follows: Group 1, comprising 6 Barrows (male animals surgically castrated within a few days of birth); Group 2, comprising 7 Boars (intact males left intact throughout the study); Group 3, comprising 6

Late Castrates (intact males left intact until approximately 135 days old at which time the animals were anesthetized and castrated surgically); and Group 4, comprising 10 intact males which were immunized with the LKT-GnRH vaccine composition at weaning (21 days old) and at approximately 135 days old.

After 42 days, the study was completed, and the animals sacrificed. Fat androstenone levels (a boar taint component) in fat specimens from animals in each experimental group were quantified by standard chemical methodology. The results are depicted in Figure 17. As can be seen in the figure, fat androstenone was similar in the barrows (Group 1), late castrates (Group 3) and immunocastrates (Group 4, treated with the LKT-GnRH vaccine), and all three groups had lower fat androstenone levels relative to the boars of Group 2.

Various aspects of the carcass composition in the experimental animals was also determined. In particular, carcass weight, back fat measurements, testicular weight (where appropriate) and bulbourethral (BU) gland length were determined in each group, and the average measurements are depicted below in Table 10. The BU gland is dependent on testosterone for maintenance of size and function.

Table 10				
Treatment Group	Carcass Weight (kg)	Back Fat (mm)	Testicular Weight (gm)	BU Length (cm)
LKT-GnRH (n=10)	90.4	24.5 (18-32)	261 (145-480)	9.6 (8.0-11.0)
Late Castrates (n=6)	88.8	24.3 (18-32)	--- ---	10.1 (8.8-12.1)
Boars (n=7)	90.3	18.3 (15-26)	641 (458-800)	14.2 (11.9-16.5)
Barrows (n=6)	83.6	28.0 (22-36)	--- ---	--- ---

As can be seen in Table 10, both testicular weight and BU gland length was significantly reduced in the immunocastrated animals of Group 4 relative to the untreated boars of Group 2, indicating that the LKT-GnRH vaccine composition was effective in reducing the levels and/or effects of serum testosterone in the vaccinated animals.

Example 15

Prediction of T-cell Epitopes in the Recombinant LKT 352 and LKT 111 Molecules

In order to predict potential T-cell epitopes in the leukotoxin polypeptide sequences employed in the LKT-GnRH chimeras of the present invention, the method proposed by Margalit and co-workers (Margalit et al., *J. Immunol* (1987) 138:2213) was performed on the amino acid sequence corresponding to numbers 1 through 199 of the LKT molecule as depicted in Table 11. Under the subject method, the amino acid sequence of the leukotoxin polypeptide sequence was compared to other sequences known to induce a T-cell response and to patterns of types of amino acids which are believed to be required for a T-

cell epitope. The results of the comparison are depicted in Table 11.

As can be seen by the predictive results thus obtained, there are several short sequences in the leukotoxin peptide which are identified as potential T-cell epitopes using the criteria suggested by Margalit et al (supra). More particularly, 9 sequences were identified as having a (Charged/Gly – Hydrophobic – Hydrophobic – Polar/Gly) sequence (indicated as pattern "1" in Table 11), and 3 sequences were identified as having a (Charged/Gly – Hydrophobic – Hydrophobic – Hydrophobic/Pro – Polar/Gly) sequence (indicated as pattern "2" in Table 11). By coupling these data with the *in vivo* anti-GnRH activity produced by both the LKT 352 and the LKT 111 carrier systems in Examples 7 and 8 above, it is indicated that critical T-cell epitopes are retained in the shortened LKT 111 molecule, and that those epitopes are likely contained within the N-terminal portion of the LKT 352 and LKT 111 molecules.

Table 11

LKT Sequence Patterns Corresponding To Potential T-cell Epitopes

LKT Amino Acid Sequences Showing Pattern "1":

GTID	(aa's 27-30)
GITG	(aa's 66-69)
GVIS	(aa's 69-72)
HVAN	(aa's 85-88)
KIVE	(aa's 93-96)
DLAG	(aa's 152-155)
KVLS	(aa's 162-165)
DAFE	(aa's 171-174)
KLVQ	(aa's 183-186)
GIID	(aa's 192-195)

LKT Amino Acid Sequence Showing Pattern "2":

RYLAN	(aa's 114-118)
KFLLN	(aa's 124-128)
KAYVD	(aa's 167-171)

Example 16

Prediction of the Physical Structure of LKT-GnRH Fusion Proteins Obtained From pCB122

In order to predict the physical structure of the B-cell epitopes of the 8 copy GnRH::LKT 111::8 copy GnRH fusion molecules obtained from the pCB122 construct, the pCB122 amino acid sequence (depicted in Figures 9-1 through 9-6) was analyzed using previously described methods for determining physical protein structure. Rost et al. (1993) *J. Mol. Biol.* 232:584-599, Rost et al. (1994) *Proteins* 19:55-72, and Rost et al. (1994) *Proteins* 20:216-226. In particular, the prediction was performed by a system of neural

networks where the input data consisted of a multiple sequence alignment. The network analysis was performed using the program MaxHom (Sander et al. (1991) *Proteins* 9:56-68, where training for the residue solvent accessibility was taken from Kabsch et al. (1983) *Biopolymers* 22:2577-2637. The neural network analysis assessed each amino acid in the pCB122 sequence, and predicted if the residue would be present as a loop, helix or exposed structure. In the prediction, the 8 copies of GnRH at the amino terminal of the pCB122 molecule were predicted to exist mainly as a loop structure, while the 8 copies of GnRH at the carboxyl terminal have a mixture of predicted structures (loop, helix and exposed residue).

. These data suggest that the enhanced immunogenicity observed with the 8 copy GnRH::LKT 111::8 copy GnRH fusion molecules obtained from the pCB122 construct may be related to the different three-dimensional structures of the GnRH antigens in the molecule.

D. Industrial Applicability

The leukotoxin-GnRH chimeras of the present invention are of use in providing immunogens that, when administered to a vertebrate host, serve to immunize the host against endogenous GnRH, which in turn acts to inhibit the reproductive function or capability of the host.

Notwithstanding the specific uses exemplified in this specification, the novel chimeric molecules disclosed herein provide a means for obtaining fusion proteins comprising more than one GnRH polypeptide, occurring in either multiple or tandem repeats, which are fused to immunogenic epitopes supplied by the leukotoxin polypeptide portion of the molecule (and in some cases by spacer

peptide sequences occurring between selected GnRH sequences). The subject chimeric proteins constructed under the present invention provide enhanced immunogenicity to the fused GnRH peptide sequences, allowing an immunized vertebrate host to mount an effective immune response toward endogenous GnRH; effecting an interruption in the synthesis and release of the two gonadotropic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) and rendering the host temporarily sterile. In this manner, the novel leukotoxin-GnRH constructs may be employed in immunosterilization vaccines to provide an alternative to invasive sterilization procedures currently practiced in domestic and farm animal husbandry.

The leukotoxin-GnRH fusion molecules can also be used to reduce the incidence of mammary tumors in mammalian subjects using vaccines comprising those molecules to block ovarian functions such as the production of the ovarian hormones estrogen and progesterone. In much the same manner, immunologically-sterilized canine and feline subjects will not develop pyometra (infection of the uterus), since the immunized animals will not produce progesterone which predisposes to that condition.

Other contemplated uses of the instant fusion molecules include population control, for example the interruption of reproduction capabilities in wild rodent populations. In this regard, the LKT-GnRH fusion molecules may be used as an alternative to population control measures currently practiced, such as poisoning and the like. The fusion products of the instant invention may also be administered in constructs having both slow and fast release components. In this manner, the need for multiple vaccinations may be avoided. Further, since the amino

acid sequence of GnRH is highly conserved among species, a single leukotoxin-GnRH fusion vaccine product may be produced which will exhibit broad cross species effectiveness.

Thus, various chimeric proteins comprising leukotoxin fused to selected GnRH polypeptides have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for a period of thirty (30) years from the date of deposit and at least five (5) years after the most recent request for the furnishing of a sample of the deposit by the depository. The organisms will be made available by the ATCC under the terms of the Budapest Treaty, which assures permanent and unrestricted availability of the cultures to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12). Upon the granting of a patent, all restrictions on the

availability to the public of the deposited cultures will be irrevocably removed.

These deposits are provided merely as convenience to those of skill in the art, and are not an admission that a deposit is required under 35 USC §112. The nucleic acid sequences of these plasmids, as well as the amino acid sequences of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the description herein. A license may be required to make, use, or sell the deposited materials, and no such license is hereby granted.

Strain No.	Deposit Date	ATCC No.
P. haemolytica serotype 1 B122	February 1, 1989	53863
pAA101 in *E. coli* JM105	February 1, 1989	67883
pAA352 in *E. coli* W1485	March 30, 1990	68283
pCB113 in *E. coli* JM105	February 1, 1995	69749
pCB111 in *E. coli* JM105	February 1, 1995	69748

Claims:

1. A chimeric protein comprising a leukotoxin polypeptide fused to first and second multimers, wherein the C-terminus of the first multimer is fused to the N-terminus of the leukotoxin polypeptide and the N-terminus of the second multimer is fused to the C-terminus of the leukotoxin polypeptide, and further wherein each of said multimers comprises more than one selected GnRH polypeptide.

2. The chimeric protein of claim 1 wherein the first and second GnRH multimers are different and comprise molecules according to the general formula $[GnRH\text{-}X\text{-}GnRH]_n$, wherein:

GnRH comprises a GnRH polypeptide;

X is selected from the group consisting of a peptide linkage, an amino acid spacer group and a leukotoxin polypeptide; and

n is an integer greater than or equal to 1.

3. The chimeric protein of claim 1 wherein the first and second GnRH multimers are the same and comprise molecules according to the general formula $[GnRH\text{-}X\text{-}GnRH]_n$, wherein:

GnRH comprises a GnRH polypeptide;

X is selected from the group consisting of a peptide linkage, an amino acid spacer group and a leukotoxin polypeptide; and

n is an integer greater than or equal to 1.

4. The chimeric protein of any of claims 2 or 3 wherein X is an amino acid spacer group having at least one helper T-cell epitope.

5. The chimeric protein of any of claims 2 or 3 wherein n is 4.

6. The chimeric protein of claim 1 wherein the leukotoxin polypeptide lacks cytotoxic activity.

7. The chimeric protein of claim 6 wherein the leukotoxin polypeptide is LKT 352.

8. The chimeric protein of any of claims 1-7 wherein the first multimer further comprises the amino acid sequence (Met-Ala-Thr-Val-Ile-Asp-Arg-Ser) fused to the N-terminus thereof.

9. The chimeric protein of claim 1 comprising the amino acid sequence depicted in Figures 9-1 through 9-6, or an amino acid sequence substantially homologous and functionally equivalent thereto.

10. A vaccine composition comprising the chimeric protein of any of claims 1-9 and a pharmaceutically acceptable vehicle.

11. A method for presenting selected GnRH multimers to a subject comprising administering to said subject an effective amount of a vaccine composition according to claim 10.

12. A DNA construct encoding a chimeric protein, wherein the chimeric protein comprises a leukotoxin polypeptide fused to first and second multimers wherein the C-terminus of the first multimer is fused to the N-terminus of the leukotoxin polypeptide and the N-terminus of the second multimer

is fused to the C-terminus of the leukotoxin polypeptide, and further wherein each of said multimers comprises more than one selected GnRH polypeptide, said DNA construct comprising:

a first nucleotide sequence encoding the first GnRH multimer; and

a second nucleotide sequence encoding the second GnRH multimer;

wherein said first and second nucleotide sequences are operably linked by a third nucleotide sequence encoding a leukotoxin polypeptide.

13. The DNA construct of claim 12 wherein the first and second GnRH multimers are different and comprise molecules according to the general formula $[GnRH\text{-}X\text{-}GnRH]_n$, wherein:

GnRH comprises a GnRH polypeptide;

X is selected from the group consisting of a peptide linkage, an amino acid spacer group and a leukotoxin polypeptide; and

n is an integer greater than or equal to 1.

14. The DNA construct of claim 12 wherein the first and second GnRH multimers are the same and comprise molecules according to the general formula $[GnRH\text{-}X\text{-}GnRH]_n$, wherein:

GnRH comprises a GnRH polypeptide;

X is selected from the group consisting of a peptide linkage, an amino acid spacer group and a leukotoxin polypeptide; and

n is an integer greater than or equal to 1.

15. The DNA construct of any of claims 13 or 14 wherein X is an amino acid spacer group having at least one helper T-cell epitope.

16. The DNA construct of any of claims 13 or 14 wherein n is 4.

17. The DNA construct of claim 12 wherein the leukotoxin polypeptide lacks cytotoxic activity.

18. The DNA construct of claim 17 wherein the leukotoxin polypeptide is LKT 352.

19. The DNA construct of any of claims 12-18 wherein the first multimer further comprises the amino acid sequence (Met-Ala-Thr-Val-Ile-Asp-Arg-Ser) fused to the N-terminus thereof.

20. The DNA construct of claim 12 wherein the chimeric protein comprises the amino acid sequence depicted in Figures 9-1 through 9-6, or an amino acid sequence substantially homologous and functionally equivalent thereto.

21. An expression cassette comprised of:

(a) the DNA construct of any of claims 12-20; and

(b) control sequences that direct the transcription of said construct whereby said construct can be transcribed and translated in a host cell.

22. A host cell transformed with the expression cassette of claim 21.

23. A method of producing a recombinant polypeptide comprising:

(a) providing a population of host cells according to claim 22; and

(b) culturing said population of cells under conditions whereby the polypeptide encoded by said expression cassette is expressed.

24. A method for reducing the incidence of mammary tumors in a mammalian subject comprising administering a therapeutically effective amount of the vaccine composition of claim 10 to said subject.

GnRH-1:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly
...CAG CAT TGG AGC TAC GGC CTG CGC CCT GGC...
...GTC GTA ACC TCG ATG CCG GAC GCG GGA CCG...

FIG. 1A

GnRH-2:

(1) (2)
[Gln His Trp Ser Tyr Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser
...CAG CAT TGG AGC TAC GGC CTG CGC CCT GGC AGC GGT TCT CAA GAT TGG AGC
...GTC GTA ACC TCG ATG CCG GAC GCG GGA CCG TCG CCA AGA GTT CTA ACC TCG
1 5 10 15

(3)
Tyr Gly Leu Arg Pro Gly Gly Ser Ser Gln His Trp Ser Tyr Gly Leu Arg
TAC GGC CTG CGT CCG GGT GGC TCT AGC CAG CAT TGG AGC TAC GGC CTG CGC
ATG CCG GAC GCA GGC CCA CCG AGA TCG GTC GTA ACC TCG ATG CCG GAC GCG
20 25 30

(4)
Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro Gly]₄
CCT GGC AGC GGT AGC CAA GAT TGG AGC TAC GGC CTG CGT CCG GGT...
GGA CCG TCG CCA TCG GTT CTA ACC TCG ATG CCG GAC GCA GGC CCA...
35 40 45 49

FIG. 1B

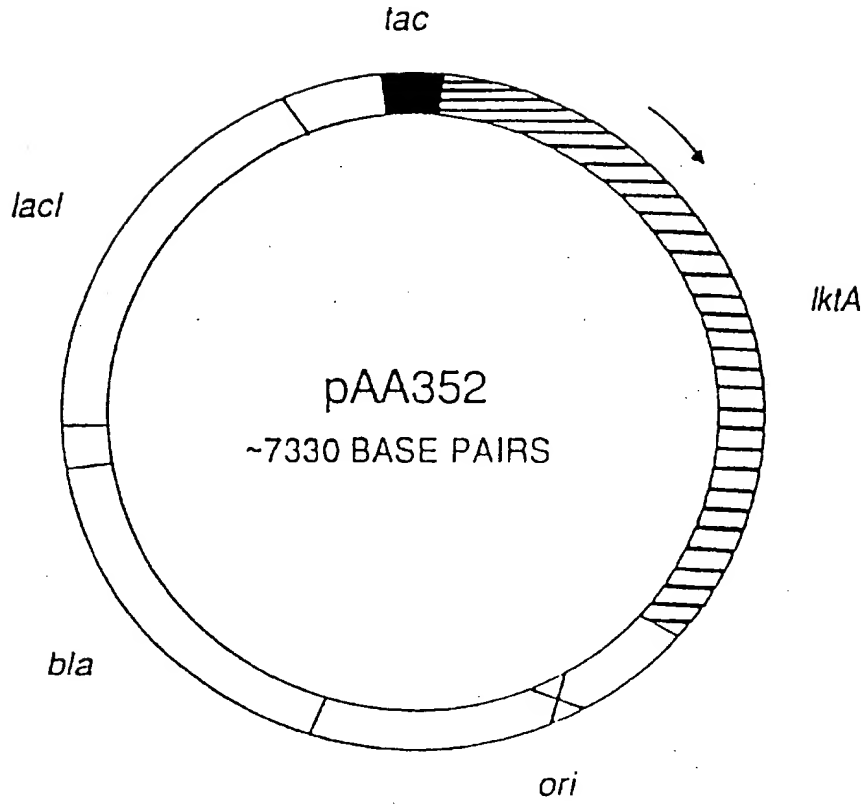

FIG. 2

FIG. 3-1

```
        10         20         30         40         50         60         70         80         90
        |          |          |          |          |          |          |          |          |
ATG GCT ACT GTT ATA GAT CTA AGC TTC CCA AAA ACT GGG GCA AAA AAA ATT ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA
TAC CGA TGA CAA TAT CTA GAT TCG AAG GGT TTT TGA CCC CGT TTT TTT TAA TAG GAG ATA TAA GGG GTT TTA ATG GTT ATA CTA TGA CTT
Met Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys Lys Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu)
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)
___a___a___VECTOR SEQUENCE_a___a___a__)

       100        110        120        130        140        150        160        170        180
        |          |          |          |          |          |          |          |          |
CAA GGT AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT
GTT CCA TTA CCA AAT GTC CTA AAT CAG TTT CGC CGG CTT CTC AAC CCC TAA CTC CAT GTT TCT CTT CTT GCG TTA TTA TAA CGT TGT CGA
Gln Gly Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala)
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)

       190        200        210        220        230        240        250        260        270
        |          |          |          |          |          |          |          |          |
CAA ACC AGT TTA GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG CGT GGC ATT GTG TTA TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG
GTT TGG TCA AAT CCG TGC TAA GTT TGG CGA TAA CCG AAT TGA CTC GCA CCG TAA CAC AAT AGG CGA GGT GTT TAA CTA TTT AAC GAT GTC
Gln Thr Ser Leu Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln)
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)
```

FIG. 3-2

```
        280         290         300         310         320         330         340         350         360
AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT GCC GAA AGC ATT GTA CAA AAT GCA AAT AAA GCC AAA ACT GTA TTA TCT GGC ATT CAA TCT
TTT TGA TTT CGT CCG GTT CGT AAT CCA AGA CGG CTT TCG TAA CAT GTT TTA CGT TTA TTT CGG TTT TGA CAT AAT AGA CCG TAA GTT AGA
Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser Gly Ile Gln Ser>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)

        370         380         390         400         410         420         430         440         450
ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG
TAA AAT CCG AGT CAT AAC CGA CCT TAC CTA AAT CTA CTC CGG AAT GTC TTA TTG TCG TTG GTT GTA CGA GAA CGA TTT CGA CCG AAC CTC
Ile Leu Gly Ser Val Leu Ala Gly Met Asp Leu Asp Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly Leu Glu>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)

        460         470         480         490         500         510         520         530         540
CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA
GAT TGT TTA AGT AAT TAA CTT TTA TAA CGA TTA AGT CAT TTT TGT GAA CTG CTT AAA CCA CTC GTT TAA TCA GTT AAA CCA AGT TTT GAT
Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)

        550         560         570         580         590         600         610         620         630
CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA
GTT TTA TAG TTT CCG AAT CCC TGA AAT CCT CTG TTT GAG TTT TTA TAG CCA CCT GAA CTA TTT CGA CCG GAA CCA AAT CTA CAA TAG AGT
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)
```

FIG. 3.3

```
      640        650        660        670        680        690        700        710        720
       |          |          |          |          |          |          |          |          |
GGG CTA TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA
CCC GAT AAT AGC CCG CGT TGT CGA CGT GAA CAT CGT CTA TTT TTA CGA AGT TGT CGA TTT TTT CAC CCA CGC CCA AAA CTT AAC CGT
Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala)
---c---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)

      730        740        750        760        770        780        790        800        810
       |          |          |          |          |          |          |          |          |
AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG
TTG GTT CAA CAA CCA TTA TAA TGG TTT CGG CAA AGA AGA ATG TAA AAT CGG GTT GCA CAA CGT CGT CCA AAT AGA AGT TGA CCC GGA CAC
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val)
---c---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)

      820        830        840        850        860        870        880        890        900
       |          |          |          |          |          |          |          |          |
GCT GCT TTA ATT GCT TCT ACT GTT TCT GTT GCG ATT AGC CCA TTA GCA TTT GCC GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA
CGA CGA AAT TAA CGA AGA TGA CAA AGA GAA CGC TAA TCG GGT AAT CGT AAA CGG CCA TAA CGG CTA TTT AAA TTA GTA CGT TTT TCA AAT
Ala Ala Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu)
---c---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)
```

FIG. 3-4

```
                910       920       930       940       950       960       970       980       990
                 |         |         |         |         |         |         |         |         |
GAG AGT TAT GCC GAA CGC TTT AAA AAA TTA GAC GGA GAT AAT TTA TTA GCA GAA TAT CAG CGG GGA ACA GGG ACT ATT GAT GCA
CTC TCA ATA CGG CTT GCG AAA TTT TTT AAT CTG CCT CTA TTA AAT AAT CGT CTT ATA GTC GCC CCT TGT CCC TGA TAA CTA CGT
Glu Ser Tyr Ala Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala)
---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)

               1000      1010      1020      1030      1040      1050      1060      1070      1080
                 |         |         |         |         |         |         |         |         |
TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC GCT ATT GGT GGT GTG TCT GCT GCA GCC GGC TCG GTT ATT GCT TCA CCG ATT GCC
AGC CAA TGA CGT TAA TTA TGG CGT AAC CGG CGA TAA CGA CCA CAC AGA CGA CGT CGG CCG AGC CAA TAA CGA AGT GGC TAA CGG
Ser Val Thr Ala Ile Asn Thr Ala Leu Ala Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala Ser Pro Ile Ala)
---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)

               1090      1100      1110      1120      1130      1140      1150      1160      1170
                 |         |         |         |         |         |         |         |         |
TTA TTA GTA TCT GGG ATT ACC GGT GTA ATT TCT ACG ATT CTG CAA TAT TCT AAA CAA GCA ATG TTT GAG CAC GTT GCA AAT AAA ATT CAT
AAT AAT CAT AGA CCC TAA TGG CCA CAT TAA AGA TGC TAA GAC GTT ATA AGA TTT GTT CGT TAC AAA CTC GTG CAA CGT TTA TTT TAA GTA
Leu Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His Val Ala Asn Lys Ile His)
---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)

               1100      1190      1200      1210      1220      1230      1240      1250      1260
                 |         |         |         |         |         |         |         |         |
AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT CAC GGT AAG AAC TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG AAT TTA CAA GAT
TTG TTT TAA CAT CTT ACC CTT TTT TTA TTA GTG CCA TTC TTG ATG AAA CTT TTA CCA ATG CTA CGG GCA ATA GAA CGC TTA AAT GTT CTA
Asn Lys Ile Val Glu Trp Glu Lys Asn Asn His Gly Lys Asn Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala Asn Leu Gln Asp)
---c---c---c---c---c---c---c---c---RECOMBINANT LEUKOTOXIN PEPTIDE-c---c---c---c---c---c---c---c---c---c---c---)
```

FIG. 3-5

```
      1270       1280       1290       1300       1310       1320       1330       1340       1350
       |          |          |          |          |          |          |          |          |
AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA CAG GCA GAA CGT GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC ATT GGT
TTA TAC TTT AAG AAT GAC TTG AAT TTG TTT CTC AAT GTC CGT CTT GCA CAG TAG CGA TAA TGA GTC GTC GTT ACC CTA TTG TTG TAA CCA
Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln Ala Glu Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly>
___C___C___C___C___C___C___C___C___C___C____RECOMBINANT LEUKOTOXIN PEPTIDE_C___C___C___C___C___C___C___C___C___C___C___)

      1360       1370       1380       1390       1400       1410       1420       1430       1440
       |          |          |          |          |          |          |          |          |
GAT TTA GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT GGT AAA GCC TAT GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC
CTA AAT CGA CCA TAA TCG GCA ATT CCA CTT TTT CAG GAA TCA CCA TTT CGG ATA CAC CTA CGC AAA CTT CTT CCG TTT GTG TAA TTT CGG
Asp Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly Lys Ala Tyr Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala>
___C___C___C___C___C___C___C___C___C___C____RECOMBINANT LEUKOTOXIN PEPTIDE_C___C___C___C___C___C___C___C___C___C___C___)

      1450       1460       1470       1480       1490       1500       1510       1520       1530
       |          |          |          |          |          |          |          |          |
GAT AAA TTA GTA CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG AGT AAT TCG GGT AAA GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG
CTA TTT AAT CAT GTC AAC CTA AGC CGT TTG CCA TAA TAA CTA CAC TCA TTA AGC CCA TTT CGC TTT TGA GTC GTA TAG AAT AAG TCT TGC
Asp Lys Leu Val Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser Asn Ser Gly Lys Ala Lys Thr Gln His Ile Leu Phe Arg Thr>
___C___C___C___C___C___C___C___C___C___C____RECOMBINANT LEUKOTOXIN PEPTIDE_C___C___C___C___C___C___C___C___C___C___C___)
```

FIG. 3-6

```
      1540        1550        1560        1570        1580        1590        1600        1610        1620
       |           |           |           |           |           |           |           |           |
CCA TTA TTG ACG CCG GGA ACA GAG CAT CGT GAA CGC GTA CAA ACA GGT AAA TAT GAA TAT ATT ACC AAG CTC AAT ATT AAC CGT GTA GAT
GGT AAT AAC TGC GGC CCT TGT CTC GTA GCA CTT GCG CAT GTT TGT CCA TTT ATA CTT ATA CTT ATA TAA TGG TTC GAG TTA TAA TTG GCA CAT CTA
Pro Leu Leu Thr Pro Gly Thr Glu His Arg Glu Val Gln Thr Gly Lys Tyr Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp)
 )___(___(___(___(___(___(___(___(___RECOMBINANT LEUKOTOXIN PEPTIDE___(___(___(___(___(___(___(___(___(___(___(

      1630        1640        1650        1660        1670        1680        1690        1700        1710
       |           |           |           |           |           |           |           |           |
AGC TGG AAA ATT ACA GAT GGT GCA GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT CAG CGT ATT GGT ATT GAA TTA GAC AAT GCT GGA
TCG ACC TTT TAA TGT CTA CGA CGT TCA AGA TGG AAA CTA AAT TGA TTG CAA CAA GTC GCA TAA CCA TAA CTT AAT CTG TTA CGA CCT
Ser Trp Lys Ile Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly)
 )___(___(___(___(___(___(___(___(___RECOMBINANT LEUKOTOXIN PEPTIDE___(___(___(___(___(___(___(___(___(___(___(

      1720        1730        1740        1750        1760        1770        1780        1790        1800
       |           |           |           |           |           |           |           |           |
AAT GTA ACT AAA ACC AAA GAA ACA AAA ATT ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT GTT GGT TCT GGT ACG ACG GAA ATT
TTA CAT TGA TTT TGG TTT CTT TGT TTT TAA TAA CGG TTT GAA CCA CTT CCA CTA CTG TTG CAT AAA CAA CCA AGA CCA TGC TGC CTT TAA
Asn Val Thr Lys Thr Lys Glu Thr Lys Ile Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe Val Gly Ser Gly Thr Thr Glu Ile)
 )___(___(___(___(___(___(___(___(___RECOMBINANT LEUKOTOXIN PEPTIDE___(___(___(___(___(___(___(___(___(___(___(

      1810        1820        1830        1840        1850        1860        1870        1880        1890
       |           |           |           |           |           |           |           |           |
GAT GGC GGT GAA GGT TAC GAC CGA GTT CAC TAT AGC CGT GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC AAA GAG ACC GAG CAA GGT
CTA CCG CCA CTT CCA ATG CTG GCT CAA GTG ATA TCG GCA CCT TTG ATA CCA CGA AAT TGA TAA CTA CGT TGG TTT CTC TGG CTC GTT CCA
Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu Gln Gly)
 )___(___(___(___(___(___(___(___(___RECOMBINANT LEUKOTOXIN PEPTIDE___(___(___(___(___(___(___(___(___(___(___(
```

FIG. 3-7

```
        1900        1910        1920        1930        1940        1950        1960        1970        1980
        |           |           |           |           |           |           |           |           |
AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC GGT AAA GCA CTA CAC GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC AAC CGT GAA GAA
TCA ATA TGG CAT TTA GCA AAG CAT CTT TGG CCA TTT CGT GAT GTG CTT CAC TGA AGT TGG GTA TGG CGT AAT CAC CCG TTG GCA CTT CTT
Ser Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu)
___c___c___c___c___c___c___c___c___c___c___RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)

        1990        2000        2010        2020        2030        2040        2050        2060        2070
        |           |           |           |           |           |           |           |           |
AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT GCC GGT TAT TAC ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC GGT ACA
TTT TAT CTT ATA GCA GTA TCG TTA TTG GTC GTG GTA CGG CCA ATA ATG TGG TTT CTA TGG AAC TTT CGA CAA CTT CTT TAA TAG CCA TGT
Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His Ala Gly Tyr Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile Gly Thr)
___c___c___c___c___c___c___c___c___c___c___RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)

        2080        2090        2100        2110        2120        2130        2140        2150        2160
        |           |           |           |           |           |           |           |           |
TCA CAT AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC TTT AAC GGT GGT GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT
AGT GTA TTG CTA TAG AAA TTT CCA TCA TTC AAG TTA CTA CGG AAA TTG CCA CCA CTA CCA CAG CTA TGA TAA CTG CCA TTG CTG CCG TTA
Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala Phe Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn)
___c___c___c___c___c___c___c___c___c___c___RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___)
```

FIG. 3-8

```
      2170       2180       2190       2200       2210       2220       2230       2240       2250
       |          |          |          |          |          |          |          |          |
GAC CGC TTA TTT GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT GGT GAT GAT TTT ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA
CTG GCG AAT AAA CCA CCA TTT CCG CTA CTA TAA GAG CTA CCA CCT TTA CCA CTA CTA AAA TAG CTA CCG CCA TTT CCG TTG CTG GAT AAT
Asp Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu)
---C---C---C---C---C---C---C---C---C---RECOMBINANT LEUKOTOXIN PEPTIDE-C---C---C---C---C---C---C---C---C---C---C---)

      2260       2270       2280       2290       2300       2310       2320       2330       2340
       |          |          |          |          |          |          |          |          |
CAC GGT GGC AAG GGC GAT GAT ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT ATT ATT ACC GAT TCT GAC GGC AAT GAT AAA TTA TCA
GTG CCA CCG TTC CCG CTA CTA TAA AAG CAA GTG GCA TTT CCG CTA CCA TTA CTA TAA TAA TGG CTA AGA CTG CCG TTA CTA TTT AAT AGT
His Gly Gly Lys Gly Asp Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp Ile Ile Thr Asp Ser Asp Gly Asn Asp Lys Leu Ser)
---C---C---C---C---C---C---C---C---C---RECOMBINANT LEUKOTOXIN PEPTIDE-C---C---C---C---C---C---C---C---C---C---C---)

      2350       2360       2370       2380       2390       2400       2410       2420       2430
       |          |          |          |          |          |          |          |          |
TTC TCT GAT TCG AAC TTA AAA GAT TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT GTC ATC ACG AAT AGC AAA AAA GAG AAA GTG ACC ATT
AAG AGA CTA AGC TTG AAT TTT CTA AAT TGT AAA CTT TTT CAA TTT GTA TTA GAA CAG TAG TGC TTA TCG TTT TTT CTC TTT CAC TGG TAA
Phe Ser Asp Ser Asn Leu Lys Asp Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile Thr Asn Ser Lys Lys Glu Lys Val Thr Ile)
---C---C---C---C---C---C---C---C---C---C---RECOMBINANT LEUKOTOXIN PEPTIDE-C---C---C---C---C---C---C---C---C---C---C---)

      2440       2450       2460       2470       2480       2490       2500       2510       2520
       |          |          |          |          |          |          |          |          |
CAA AAC TGG TTC CGA GAG GCT GAT TTT GCT AAA GAA GTG CCT AAT TAT AAA GCA ACT AAA GAT GAG AAA ATC GAA GAA ATC ATC GGT CAA
GTT TTG ACC AAG GCT CTC CGA CTA AAA CGA TTT CTT CAC GGA TTA ATA TTT CGT TGA TTT CTA CTC TTT TAG CTT CTT TAG TAG CCA GTT
Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu Ile Ile Gly Gln)
---C---C---C---C---C---C---C---C---C---C---RECOMBINANT LEUKOTOXIN PEPTIDE-C---C---C---C---C---C---C---C---C---C---C---)
```

FIG. 3-9

```
      2530        2540        2550        2560        2570        2580        2590        2600        2610
      |    |      |    |      |    |      |    |      |    |      |    |      |    |      |    |      |    |
AAT GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA GTT GTT
TTA CCG CTC GCC TAG TGG AGT TTC GTT CAA CTA CTA GAA TAG CGT TTT CCA TTG CCG TTT TAA TGG GTT CTA CTC GAT AGT TTT CAA CAA
Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys Val Val)
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___c___)

      2620        2630        2640        2650        2660        2670        2680        2690        2700
      |    |      |    |      |    |      |    |      |    |      |    |      |    |      |    |      |    |
GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT
CTA TTG ATA CTT AAC GAG TTT GTA TCG TTT TTA CAC TGT TTG TCG AAT CTA TTC AAT TAG AGT AGA CAT TCA CGT AAA TGG AGC AGA TTA
Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn)
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___c___)

      2710        2720        2730        2740        2750        2760        2770        2780        2790
      |    |      |    |      |    |      |    |      |    |      |    |      |    |      |    |      |    |
GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT TTA TCT TCT CTT CAA TTT GCT AGG GGA TCC TAG CTAGCTAGCCATG
CTA AGC TCT TTA CAT AAT CAC CGA GGT TGA AGT TAC AAC CTA GTT TCA AAT AGA AGA GAA GTT AAA CGA TCC CCT AGG ATC GATCGATCGGTAC
Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met Leu Asp Gln Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser End)
___c___c___c___c___c___c___c___c___c__RECOMBINANT LEUKOTOXIN PEPTIDE___c___c___c___c___c___c___c___c___c___c___)
                                                                                                  _b___VECTOR SEQUENCE_____)
```

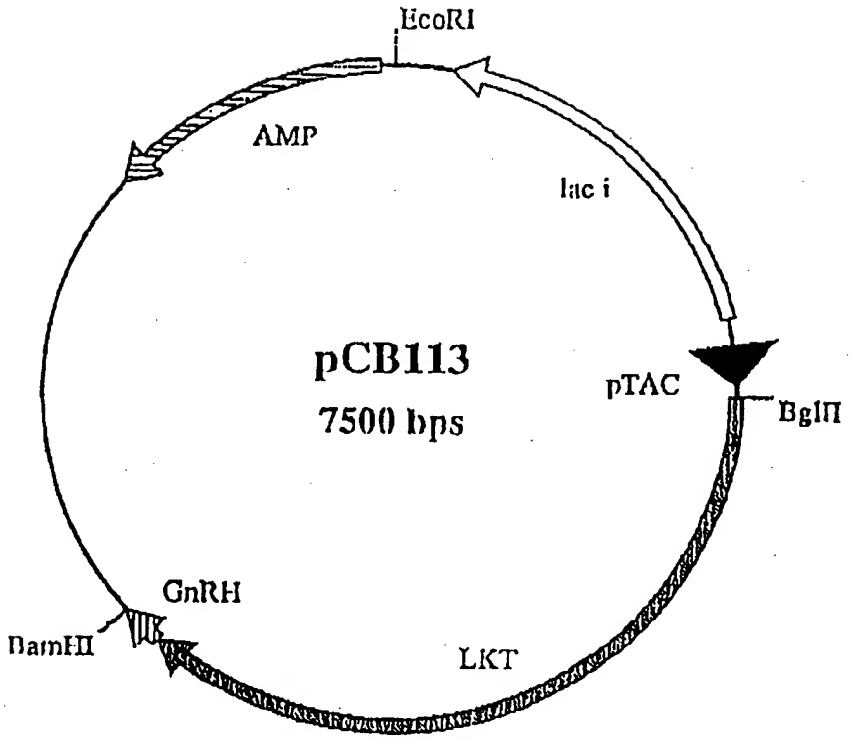

FIG. 4

```
          10          20          30          40
ATG GCT ACT GTT ATA GAT CTA AGC TTC CCA AAA ACT GGG GCA AAA
MET Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys

     50          60          70          80          90
AAA ATT ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA
Lys Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu

          100         110         120         130
CAA GGT AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG
Gln Gly Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu

    140         150         160         170         180
GGG ATT GAG GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT
Gly Ile Glu Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala

          190         200         210         220
CAA ACC AGT TTA GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG
Gln Thr Ser Leu Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu

    230         240         250         260         270
CGT GGC ATT GTG TTA TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG
Arg Gly Ile Val Leu Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln

          280         290         300         310

AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT GCC GAA AGC ATT GTA
Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser Ala Glu Ser Ile Val

    320         330         340         350         360
CAA AAT GCA AAT AAA GCC AAA ACT GTA TTA TCT GGC ATT CAA TCT
Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser Gly Ile Gln Ser

          370         380         390         400
ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT GAG GCC TTA
Ile Leu Gly Ser Val Leu Ala Gly MET Asp Leu Asp Glu Ala Leu
```

FIG. 5-1

```
    410         420         430         440         450
CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG
Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly Leu Glu

        460         470         480         490
CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA
Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr

    500         510         520         530         540
CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA
Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu

        550         560         570         580
CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn

    590         600         610         620         630
ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA
Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser

        640         650         660         670
GGG CTA TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA
Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys

    680         690         700         710         720
AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA
Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala

        730         740         750         760
AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile

    770         780         790         800         810
TTA GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG
Leu Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val
```

FIG. 5-2

```
          820         830         840         850
GCT GCT TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA
Ala Ala Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu

   860         870         880         890         900
GCA TTT GCC GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA
Ala Phe Ala Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu

          910         920         930         940
GAG AGT TAT GCC GAA CGC TTT AAA AAA TTA GGC TAT GAC GGA GAT
Glu Ser Tyr Ala Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp

   950         960         970         980         990
AAT TTA TTA GCA GAA TAT CAG CGG GGA ACA GGC ACT ATT GAT GCA
Asn Leu Leu Ala Glu Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala

         1000        1010        1020        1030
TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC GCT ATT GCT GGT GGT
Ser Val Thr Ala Ile Asn Thr Ala Leu Ala Ala Ile Ala Gly Gly

  1040        1050        1060        1070        1080
GTG TCT GCT GCT GCA GCC GGC TCG GTT ATT GCT TCA CCG ATT GCC
Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala Ser Pro Ile Ala

         1090        1100        1110        1120
TTA TTA GTA TCT GGG ATT ACC GGT GTA ATT TCT ACG ATT CTG CAA
Leu Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr Ile Leu Gln

  1130        1140        1150        1160        1170
TAT TCT AAA CAA GCA ATG TTT GAG CAC GTT GCA AAT AAA ATT CAT
Tyr Ser Lys Gln Ala MET Phe Glu His Val Ala Asn Lys Ile His

         1180        1190        1200        1210
AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT CAC GGT AAG AAC TAC
Asn Lys Ile Val Glu Trp Glu Lys Asn Asn His Gly Lys Asn Tyr
```

FIG. 5-3

```
1220        1230        1240        1250        1260
TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG AAT TTA CAA GAT
Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala Asn Leu Gln Asp

      1270        1280        1290        1300
AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA CAG GCA GAA
Asn MET Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln Ala Glu

1310        1320        1330        1340        1350
CGT GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC ATT GGT
Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly

      1360        1370        1380        1390
GAT TTA GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT GGT
Asp Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly

1400        1410        1420        1430        1440
AAA GCC TAT GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC
Lys Ala Tyr Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala

      1450        1460        1470        1480
GAT AAA TTA GTA CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG
Asp Lys Leu Val Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val

1490        1500        1510        1520        1530
AGT AAT TCG GGT AAA GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG
Ser Asn Ser Gly Lys Ala Lys Thr Gln His Ile Leu Phe Arg Thr

      1540        1550        1560        1570
CCA TTA TTG ACG CCG GGA ACA GAG CAT CGT GAA CGC GTA CAA ACA
Pro Leu Leu Thr Pro Gly Thr Glu His Arg Glu Arg Val Gln Thr

1580        1590        1600        1610        1620
GGT AAA TAT GAA TAT ATT ACC AAG CTC AAT ATT AAC CGT GTA GAT
Gly Lys Tyr Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp
```

FIG. 5-4

```
          1630        1640        1650        1660
AGC TGG AAA ATT ACA GAT GGT GCA GCA AGT TCT ACC TTT GAT TTA
Ser Trp Lys Ile Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu

  1670        1680        1690        1700        1710
ACT AAC GTT GTT CAG CGT ATT GGT ATT GAA TTA GAC AAT GCT GGA
Thr Asn Val Val Gln Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly

          1720        1730        1740        1750
AAT GTA ACT AAA ACC AAA GAA ACA AAA ATT ATT GCC AAA CTT GGT
Asn Val Thr Lys Thr Lys Glu Thr Lys Ile Ile Ala Lys Leu Gly

  1760        1770        1780        1790        1800
GAA GGT GAT GAC AAC GTA TTT GTT GGT TCT GGT ACG ACG GAA ATT
Glu Gly Asp Asp Asn Val Phe Val Gly Ser Gly Thr Thr Glu Ile

          1810        1820        1830        1840
GAT GGC GGT GAA GGT TAC GAC CGA GTT CAC TAT AGC CGT GGA AAC
Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser Arg Gly Asn

  1850        1860        1870        1880        1890
TAT GGT GCT TTA ACT ATT GAT GCA ACC AAA GAG ACC GAG CAA GGT
Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu Gln Gly

          1900        1910        1920        1930
AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC GGT AAA GCA CTA CAC
Ser Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu His

  1940        1950        1960        1970        1980
GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC AAC CGT GAA GAA
Glu Val Thr Ser Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu

          1990        2000        2010        2020
AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT GCC GGT TAT
Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His Ala Gly Tyr
```

FIG. 5-5

```
2030        2040        2050        2060        2070
TAC ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC GGT ACA
Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile Gly Thr

      2080        2090        2100        2110
TCA CAT AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC TTT
Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala Phe

2120        2130        2140        2150        2160
AAC GGT GGT GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT
Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn

      2170        2180        2190        2200
GAC CGC TTA TTT GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA
Asp Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly

2210        2220        2230        2240        2250
AAT GGT GAT GAT TTT ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA
Asn Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu

      2260        2270        2280        2290
CAC GGT GGC AAG GGC GAT GAT ATT TTC GTT CAC CGT AAA GGC GAT
His Gly Gly Lys Gly Asp Asp Ile Phe Val His Arg Lys Gly Asp

2300        2310        2320        2330        2340
GGT AAT GAT ATT ATT ACC GAT TCT GAC GGC AAT GAT AAA TTA TCA
Gly Asn Asp Ile Ile Thr Asp Ser Asp Gly Asn Asp Lys Leu Ser

      2350        2360        2370        2380
TTC TCT GAT TCG AAC TTA AAA GAT TTA ACA TTT GAA AAA GTT AAA
Phe Ser Asp Ser Asn Leu Lys Asp Leu Thr Phe Glu Lys Val Lys

2390        2400        2410        2420        2430
CAT AAT CTT GTC ATC ACG AAT AGC AAA AAA GAG AAA GTG ACC ATT
His Asn Leu Val Ile Thr Asn Ser Lys Lys Glu Lys Val Thr Ile
```

FIG. 5-6

```
          2440        2450        2460        2470
CAA AAC TGG TTC CGA GAG GCT GAT TTT GCT AAA GAA GTG CCT AAT
Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala Lys Glu Val Pro Asn

 2480        2490        2500        2510        2520
TAT AAA GCA ACT AAA GAT GAG AAA ATC GAA GAA ATC ATC GGT CAA
Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu Ile Ile Gly Gln

          2530        2540        2550        2560
AAT GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT CTT ATC GCA
Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp Leu Ile Ala

 2570        2580        2590        2600        2610
AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA GTT GTT
Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys Val Val

          2620        2630        2640        2650
GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC AGC
Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn Ser

 2660        2670        2680        2690        2700
TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT
Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn

          2710        2720        2730        2740
GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA
Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser MET Leu Asp Gln

 2750        2760        2770        2780        2790
AGT TTA TCT TCT CTT CAA TTT GCT AGG GGA TCT CAG CAT TGG AGC
Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser Gln His Trp Ser

          2800        2810        2820        2830
TAC GGC CTG CGC CCT GGC AGC GGT TCT CAA GAT TGG ACC TAC GGC
Tyr Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly
```

FIG. 5-7

```
  2840          2850          2860          2870          2880
CTG CGT CCG GGT GGC TCT AGC CAG CAT TGG AGC TAC GGC CTG CGC
Leu Arg Pro Gly Gly Ser Ser Gln His Trp Ser Tyr Gly Leu Arg

          2890          2900          2910          2920
CCT GGC AGC GGT AGC CAA GAT TGG AGC TAC GGC CTG CGT CCG GGT
Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro Gly

  2930
GGA TCC TAG
Gly Ser ---
```

FIG. 5-8

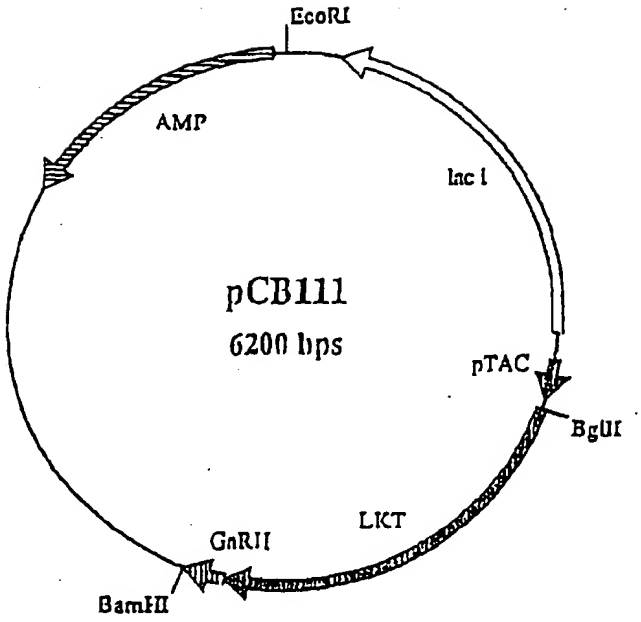

FIG. 6

```
          10         20         30         40
ATG GCT ACT GTT ATA GAT CTA AGC TTC CCA AAA ACT GGG GCA AAA
MET Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys

    50         60         70         80         90
AAA ATT ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA
Lys Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu

         100        110        120        130
CAA GGT AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG
Gln Gly Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu

   140        150        160        170        180
GGG ATT GAG GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT
Gly Ile Glu Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala

         190        200        210        220
CAA ACC AGT TTA GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG
Gln Thr Ser Leu Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu

   230        240        250        260        270
CGT GGC ATT GTG TTA TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG
Arg Gly Ile Val Leu Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln

         280        290        300        310
AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT GCC GAA AGC ATT GTA
Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser Ala Glu Ser Ile Val

   320        330        340        350        360
CAA AAT GCA AAT AAA GCC AAA ACT GTA TTA TCT GGC ATT CAA TCT
Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser Gly Ile Gln Ser

         370        380        390        400
ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT GAG GCC TTA
Ile Leu Gly Ser Val Leu Ala Gly MET Asp Leu Asp Glu Ala Leu
```

FIG. 7-1

```
    410         420         430         440         450
CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG
Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly Leu Glu

            460         470         480         490
CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA
Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr

    500         510         520         530         540
CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA
Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu

            550         560         570         580
CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn

    590         600         610         620         630
ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA
Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser

            640         650         660         670
GGG CTA TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA
Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys

    680         690         700         710         720
AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA
Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala

            730         740         750         760
AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile
```

FIG. 7-2

```
    770         780         790         800         810
TTA GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG
Leu Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val

            820         830         840         850
GCT GCT TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA
Ala Ala Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu

    860         870         880         890         900
GCA TTT GCC GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA
Ala Phe Ala Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu

            910         920         930         940
GAG AGT TAT GCC GAA CGC TTT AAA AAA TTA GGC TAT GAC GGA GAT
Glu Ser Tyr Ala Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp

    950         960         970         980         990
AAT TTA TTA GCA GAA TAT CAG CGG GGA ACA GGG ACT ATT GAT GCA
Asn Leu Leu Ala Glu Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala

           1000        1010        1020        1030
TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC GCT ATT GCT GGT GGT
Ser Val Thr Ala Ile Asn Thr Ala Leu Ala Ala Ile Ala Gly Gly

   1040        1050        1060        1070        1080
GTG TCT GCT GCT GCA GCC AAC TTA AAA GAT TTA ACA TTT GAA AAA
Val Ser Ala Ala Ala Ala Asn Leu Lys Asp Leu Thr Phe Glu Lys

           1090        1100        1110        1120
GTT AAA CAT AAT CTT GTC ATC ACG AAT AGC AAA AAA GAG AAA GTG
Val Lys His Asn Leu Val Ile Thr Asn Ser Lys Lys Glu Lys Val

   1130        1140        1150        1160        1170
ACC ATT CAA AAC TGG TTC CGA GAG GCT GAT TTT GCT AAA GAA GTG
Thr Ile Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala Lys Glu Val
```

FIG. 7-3

```
          1180        1190        1200        1210
CCT AAT TAT AAA GCA ACT AAA GAT GAG AAA ATC GAA GAA ATC ATC
Pro Asn Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu Ile Ile

  1220        1230        1240        1250        1260
GGT CAA AAT GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT CTT
Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp Leu

          1270        1280        1290        1300
ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA
Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys

  1310        1320        1330        1340        1350
GTT GTT GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA
Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr

          1360        1370        1380        1390
AAC AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG
Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser

  1400        1410        1420        1430        1440
TCT AAT GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG
Ser Asn Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser MET Leu

          1450        1460        1470        1480
GAT CAA AGT TTA TCT TCT CTT CAA TTT GCT AGG GGA TCT CAG CAT
Asp Gln Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser Gln His

  1490        1500        1510        1520        1530
TGG AGC TAC GGC CTG CGC CCT GGC AGC GGT TCT CAA GAT TGG AGC
Trp Ser Tyr Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser
```

FIG. 7-4

```
          1540          1550          1560          1570
TAC GGC CTG CGT CCG GGT GGC TCT AGC CAG CAT TGG AGC TAC GGC
Tyr Gly Leu Arg Pro Gly Gly Ser Ser Gln His Trp Ser Tyr Gly

  1580          1590          1600          1610          1620
CTG CGC CCT GGC AGC GGT AGC CAA GAT TGG AGC TAC GGC CTG CGT
Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg

          1630
CCG GGT GGA TCC TAG
Pro Gly Gly Ser ---
```

FIG. 7-5

```
            [Nae1]                                          [BstB1]
..GCT GCA GCC|GGC TCG GTT ATT....TTC TCT GAT TCG|AAC TTA AAA..
..CGA CGT CGG|CCG AGC CAA TAA...AAG AGA CTA AGC|TTG AAT TTT..
..Ala Ala Ala|Gly Ser Val Ile...Phe Ser Asp Ser|Asn Leu Lys...
          351                                          785
```

FIG. 8-1

```
..GCT GCA GCC  AAC TTA AAA..
..CGA CGT CGG  TTG AAT TTT..
..Ala Ala Ala  Asn Leu Lys...
          351  785
```

FIG. 8-2

```
          10          20          30          40
ATG GCT ACT GTT ATA GAT CGA TCT CAG CAT TGG AGC TAC GGC CTG
MET Ala Thr Val Ile Asp Arg Ser Gln His Trp Ser Tyr Gly Leu

     50          60          70          80          90
CGC CCT GGC AGC GGT TCT CAA GAT TGG AGC TAC GGC CTG CGT CCG
Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro

          100         110         120         130
GGT GGC TCT AGC CAG CAT TGG AGC TAC GGC CTG CGC CCT GGC AGC
Gly Gly Ser Ser Gln His Trp Ser Tyr Gly Leu Arg Pro Gly Ser

    140         150         160         170         180
GGT AGC CAA GAT TGG AGC TAC GGC CTG CGT CCG GGT GGA TCT CAG
Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro Gly Gly Ser Gln

          190         200         210         220
CAT TGG AGC TAC GGC CTG CGC CCT GGC AGC GGT TCT CAA GAT TGG
His Trp Ser Tyr Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp

    230         240         250         260         270
AGC TAC GGC CTG CGT CCG GGT GGC TCT AGC CAG CAT TGG AGC TAC
Ser Tyr Gly Leu Arg Pro Gly Gly Ser Ser Gln His Trp Ser Tyr

          280         290         300         310
GGC CTG CGC CCT GGC AGC GGT AGC CAA GAT TGG AGC TAC GGC CTG
Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu
```

FIG. 9-1

```
   320          330          340          350          360
CGT CCG GGT GGA TCT AGC TTC CCA AAA ACT GGG GCA AAA AAA ATT
Arg Pro Gly Gly Ser Ser Phe Pro Lys Thr Gly Ala Lys Lys Ile

          370          380          390          400
ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA CAA GGT
Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly

   410          420          430          440          450
AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT
Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile

          460          470          480          490
GAG GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT CAA ACC
Glu Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr

   500          510          520          530          540
AGT TTA GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG CGT GGC
Ser Leu Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu Arg Gly

          550          560          570          580
ATT GTG TTA TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG AAA ACT
Ile Val Leu Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln Lys Thr

   590          600          610          620          630
AAA GCA GGC CAA GCA TTA GGT TCT GCC GAA AGC ATT GTA CAA AAT
Lys Ala Gly Gln Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn

          640          650          660          670
GCA AAT AAA GCC AAA ACT GTA TTA TCT GGC ATT CAA TCT ATT TTA
Ala Asn Lys Ala Lys Thr Val Leu Ser Gly Ile Gln Ser Ile Leu
```

FIG. 9-2

```
   680         690         700         710         720
GGC TCA GTA TTG GCT CGA ATC GAT TTA GAT GAG GCC TTA CAG AAT
Gly Ser Val Leu Ala Gly MET Asp Leu Asp Glu Ala Leu Gln Asn

          730         740         750         760
AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG CTA ACA
Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly Leu Glu Leu Thr

   770         780         790         800         810
AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA CTT GAC
Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr Leu Asp

          820         830         840         850
GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA CAA AAT
Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu Gln Asn

   860         870         880         890         900
ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC GGT
Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile Gly

          910         920         930         940
GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA GGG CTA
Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser Gly Leu

   950         960         970         980         990
TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT
Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala

         1000        1010        1020        1030
TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA AAC CAA
Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala Asn Gln

  1040        1050        1060        1070        1080
GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA GCC
Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu Ala
```

FIG. 9-3

```
          1090         1100         1110         1120
CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG GCT GCT
Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala

  1130         1140         1150         1160         1170
TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT
Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe

          1180         1190         1200         1210
GCC GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA GAG AGT
Ala Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu Glu Ser

  1220         1230         1240         1250         1260
TAT GCC GAA CGC TTT AAA AAA TTA GGC TAT GAC GGA GAT AAT TTA
Tyr Ala Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu

          1270         1280         1290         1300
TTA GCA GAA TAT CAG CGG GGA ACA GGG ACT ATT GAT GCA TCG GTT
Leu Ala Glu Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala Ser Val

  1310         1320         1330         1340         1350
ACT GCA ATT AAT ACC GCA TTG GCC GCT ATT GCT GGT GGT GTG TCT
Thr Ala Ile Asn Thr Ala Leu Ala Ala Ile Ala Gly Gly Val Ser

          1360         1370         1380         1390
GCT GCT GCA GCC GAT TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT
Ala Ala Ala Ala Asp Leu Thr Phe Glu Lys Val Lys His Asn Leu

  1400         1410         1420         1430         1440
GTC ATC ACG AAT AGC AAA AAA GAG AAA GTG ACC ATT CAA AAC TGG
Val Ile Thr Asn Ser Lys Lys Glu Lys Val Thr Ile Gln Asn Trp
```

FIG. 9-4

```
          1450          1460          1470          1480
TTC CGA GAG GCT GAT TTT GCT AAA GAA GTG CCT AAT TAT AAA GCA
Phe Arg Glu Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala

  1490          1500          1510          1520          1530
ACT AAA GAT GAG AAA ATC GAA GAA ATC ATC GGT CAA AAT GGC GAG
Thr Lys Asp Glu Lys Ile Glu Glu Ile Ile Gly Gln Asn Gly Glu

          1540          1550          1560          1570
CGG ATC ACC TCA AAG CAA GTT GAT GAT CTT ATC GCA AAA GGT AAC
Arg Ile Thr Ser Lys Gln Val Asp Asp Leu Ile Ala Lys Gly Asn

  1580          1590          1600          1610          1620
GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA GTT GTT GAT AAC TAT
Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys Val Val Asp Asn Tyr

          1630          1640          1650          1660
GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC AGC TTA GAT AAG
Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn Ser Leu Asp Lys

  1670          1680          1690          1700          1710
TTA ATC TCA TCT GTA ACT GCA TTT ACC TCG TCT AAT GAT TCG AGA
Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn Asp Ser Arg

          1720          1730          1740          1750
AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT TTA TCT
Asn Val Leu Val Ala Pro Thr Ser MET Leu Asp Gln Ser Leu Ser

  1760          1770          1780          1790          1800
TCT CTT CAA TTT GCT AGG GGA TCT CAG CAT TGG AGC TAC GGC CTG
Ser Leu Gln Phe Ala Arg Gly Ser Gln His Trp Ser Tyr Gly Leu
```

FIG. 9-5

```
          1810         1820         1830         1840
CGC CCT GGC AGC GGT TCT CAA GAT TGG AGC TAC GGC CTG CGT CCG
Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro

  1850         1860         1870         1880         1890
GGT GGC TCT AGC CAG CAT TGG AGC TAC GGC CTG CGC CCT GGC AGC
Gly Gly Ser Ser Gln His Trp Ser Tyr Gly Leu Arg Pro Gly Ser

          1900         1910         1920         1930
GGT AGC CAA GAT TGG AGC TAC GGC CTG CGT CCG GGT GGA TCT CAG
Gly Ser Gln Asp Trp Ser Tyr Gly Leu Arg Pro Gly Gly Ser Gln

  1940         1950         1960         1970         1980
CAT TGG AGC TAC GGC CTG CGC CCT GGC AGC GGT TCT CAA GAT TGG
His Trp Ser Tyr Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp

          1990         2000         2010         2020
AGC TAC GGC CTG CGT CCG GGT GGC TCT AGC CAG CAT TGG AGC TAC
Ser Tyr Gly Leu Arg Pro Gly Gly Ser Ser Gln His Trp Ser Tyr

  2030         2040         2050         2060         2070
GGC CTG CGC CCT GGC AGC GGT AGC CAA GAT TGG AGC TAC GGC CTG
Gly Leu Arg Pro Gly Ser Gly Ser Gln Asp Trp Ser Tyr Gly Leu

          2080         2090         2100
CGT CCG GGT GGA TCC TAG CTA GCT AGC CAT CG
Arg Pro Gly Gly Ser --- Leu Ala Ser His
```

FIG. 9-6

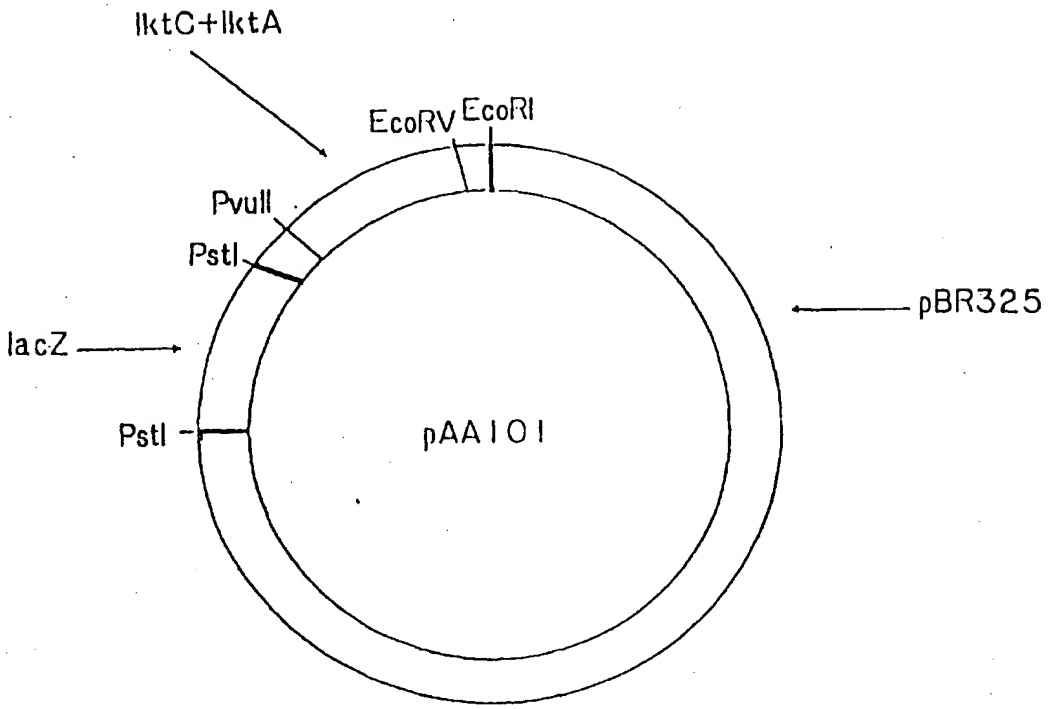

FIG. 10

```
1    MGTRLTTLSNGLKNTLTATKSGLHKAGQSLTQAGSSLKTGAKKIILYIPQNYQYDTEQGN
61   GLQDLVKAAEELGIEVQREERNNIATAQTSLGTIQTAIGLTERGIVLSAPQIDKLLQKTK
121  AGQALGSAESIVQNANKAKTVLSGIQSILGSVLAGMDLDEALQNNSNQHALAKAGLELTN
181  SLIENIANSVKTLDEFGEQISQFGSKLQNIKGLGTLGDKLKNIGGLDKAGLGLDVISGLL
241  SGATAALVLADKNASTAKKVGAGFELANQVVGNITKAVSSYILAQRVAAGLSSTGPVAAL
301  IASTVSLAISPLAFAGIADKFNHAKSLESYAERFKKLGYDGDNLLAEYQRGTGTIDASVT
361  AINTALAAIAGGVSAAAGRRIRGIPGDPVVLQRRDWENPGVTQLNRLAAHPPFASWRNSE
421  EARTDRPSQQLRSLNGEWRFAWFPAPEAVPESWLECDLPEADTVVVPSNWQMHGYDAPIY
481  TNVTYPITVNPPFVPTENPTGCYSLTFNVDESWLQEGQTRIIFDGVNSAFHLWCNGRWVG
541  YGQDSRLPSEFDLSAFLRAGENRLAVMVLRWSDGSYLEDQDMWRMSGIFRDVSLLHKPTT
601  QISDFHVATRFNDDFSRAVLEAEVQMCGELRDYLRVTVSLWQGETQVASGTAPFGGEIID
661  ERGGYADRVTLRLNVENPKLWSAEIPNLYRAVVELHTADGTLIEAEACDVGFREVRIENG
721  LLLLNGKPLLIRGVNRHEHHPLHGQVMDEQTMVQDILLMKQNNFNAVRCSHYPNHPLWYT
781  LCDRYGLYVVDEANIETHGMVPMNRLTDDPRWLPAMSERVTRMVQRDRNHPSVIIWSLGN
841  ESGHGANHDALYRWIKSVDPSRPVQYEGGGADTTATDIICPMYARVDEDQPFPAVPKWSI
901  KKWLSLPGETRPLILCEYAHAMGNSLGGFAKYWQAFRQYPRLQGGFVWDWVDQSLIKYDE
961  NGNPWSAYGGDFGDTPNDRQFCMNGLVFADRTPHPALTEAKHQQQFFQFRLSGQTIEVTS
1021 EYLFRHSDNELLHWMVALDGKPLASGEVPLDVAPQGKQLIELPELPQPESAGQLWLTVRV
1081 VQPNATAWSEAGHISAWQQWRLAENLSVTLPAASHAIPHLTTSEMDFCIELGNKRWQFNR
1141 QSGFLSQMWIGDKKQLLTPLRDQFTRAPLDNDIGVSEATRIDPNAWVERWKAAGHYQAEA
1201 ALLQCTADTLADAVLITTAHAWQHQGKTLFISRKTYRIDGSGQMAITVDVEVASDTPHPA
1261 RIGLNCQLAQVAERVNWLGLGPQENYPDRLTAACFDRWDLPLSDMYTPYVFPSENGLRCG
1321 TRELNYGPHQWRGDFQFNISRYSQQQLMETSHRHLLHAEEGTWLNIDGFHMGIGGDDSWS
1381 PSVSAEFQLSAGRYHYQLVWCQK
```

FIG. 11

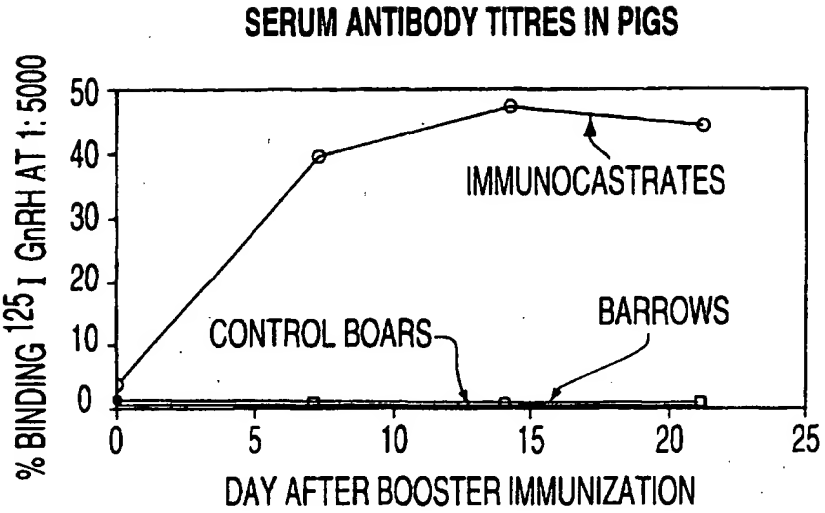
SERUM ANTIBODY TITRES IN PIGS
% BINDING ^{125}I GnRH AT 1:5000
50
40
30
20
10
0
IMMUNOCASTRATES
BARROWS
CONTROL BOARS
0
5
10
15
20
25
DAY AFTER BOOSTER IMMUNIZATION

FIG. 12

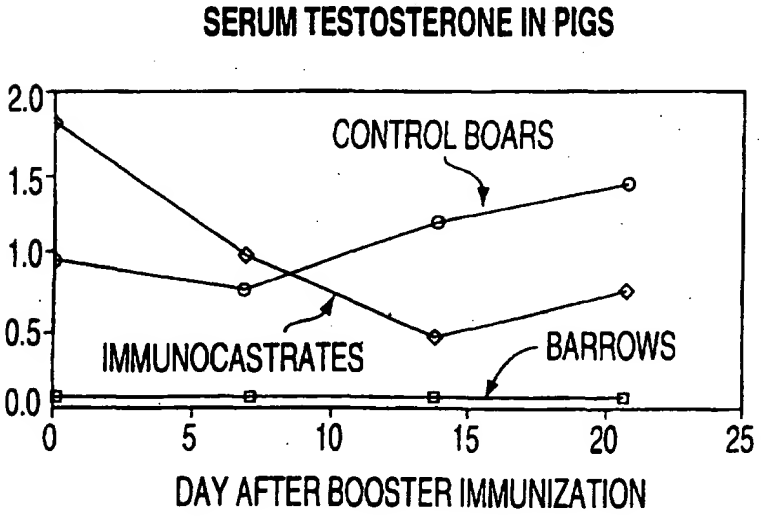
SERUM TESTOSTERONE IN PIGS
2.0
1.5
1.0
0.5
0.0
CONTROL BOARS
IMMUNOCASTRATES
BARROWS
0
5
10
15
20
25
DAY AFTER BOOSTER IMMUNIZATION

FIG. 13

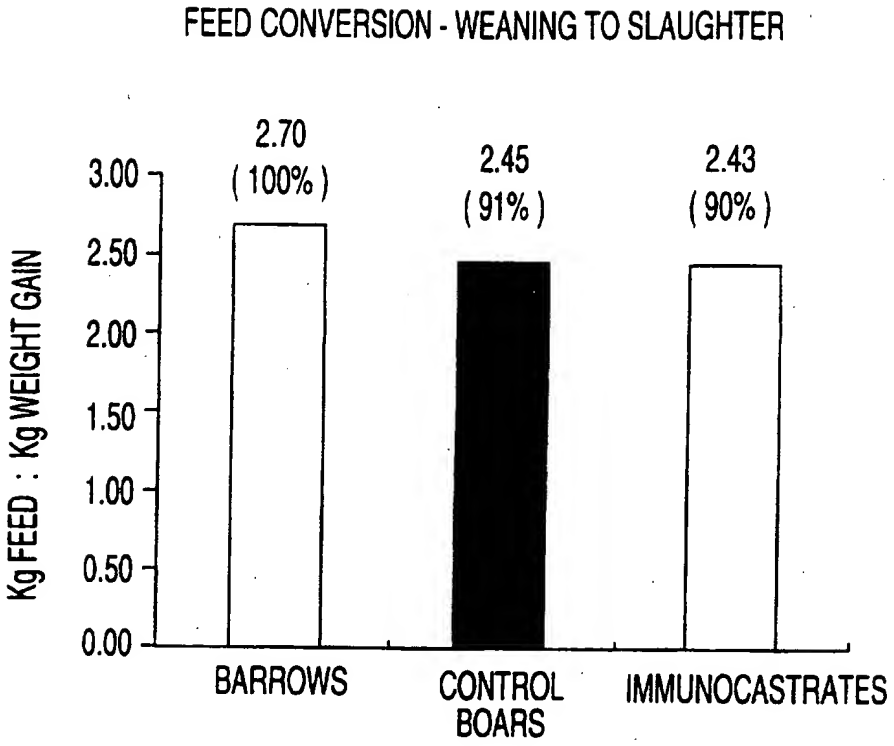
FEED CONVERSION - WEANING TO SLAUGHTER
Kg FEED : Kg WEIGHT GAIN
3.00
2.50
2.00
1.50
1.00
0.50
0.00
2.70
(100%)
2.45
(91%)
2.43
(90%)
BARROWS
CONTROL BOARS
IMMUNOCASTRATES

FIG. 14

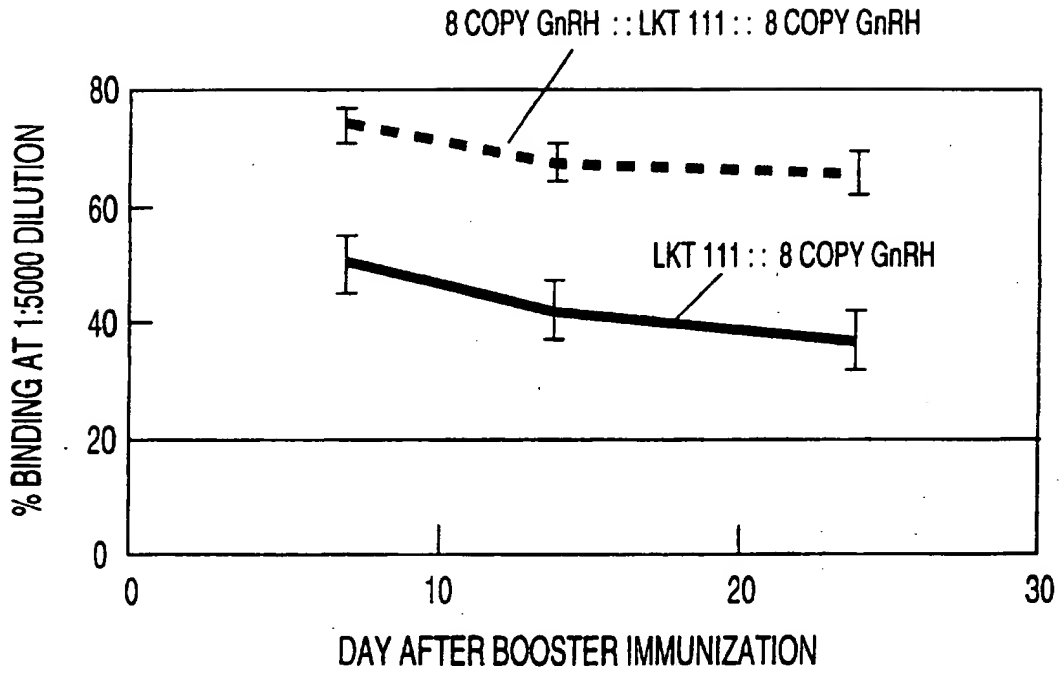
8 COPY GnRH : : LKT 111 : : 8 COPY GnRH
LKT 111 : : 8 COPY GnRH
% BINDING AT 1:5000 DILUTION
80
60
40
20
0
0
10
20
30
DAY AFTER BOOSTER IMMUNIZATION

FIG. 15

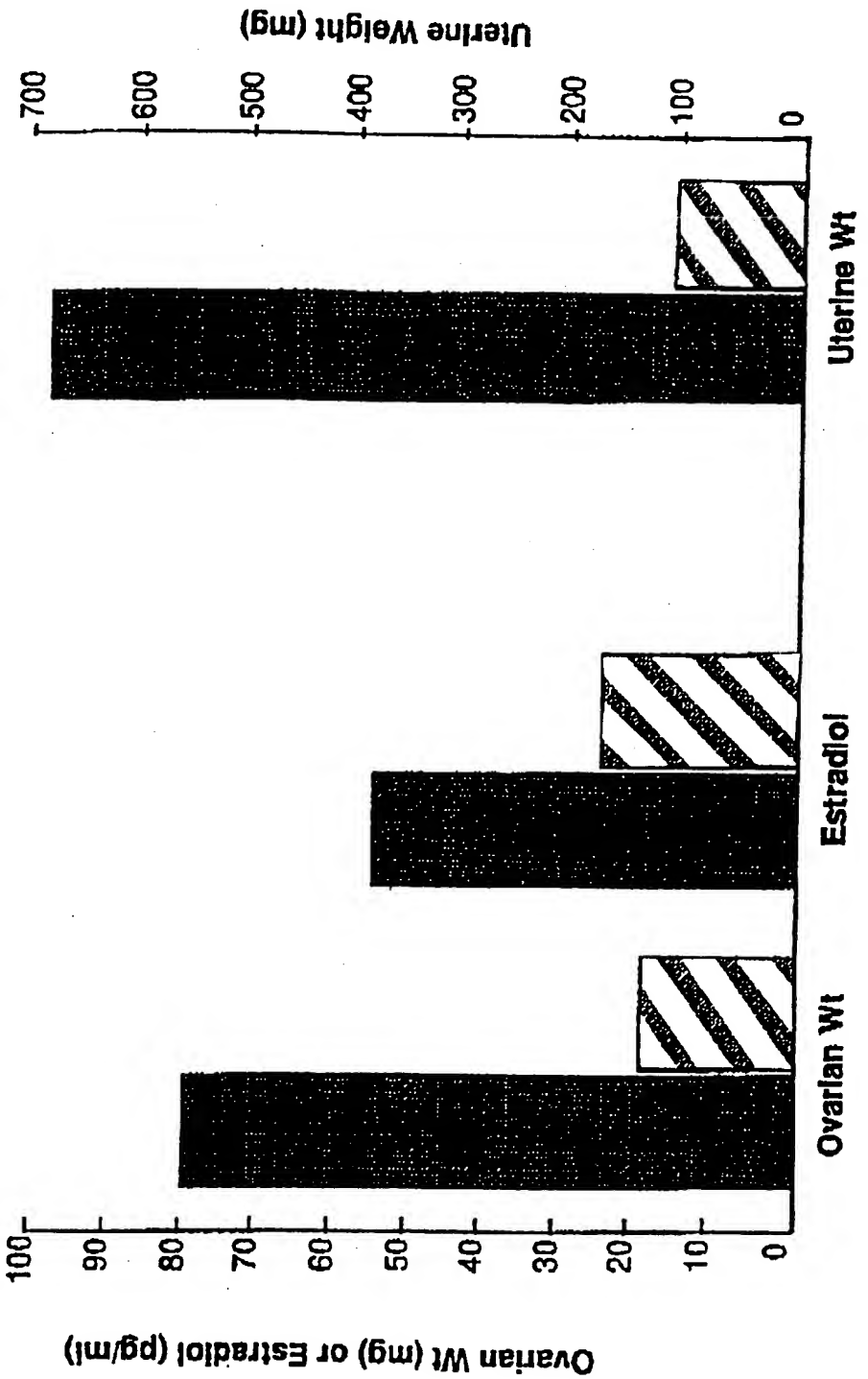
Ovarian Wt (mg) or Estradiol (pg/ml)
0
10
20
30
40
50
60
70
80
90
100
Uterine Weight (mg)
0
100
200
300
400
500
600
700
Ovarian Wt
Estradiol
Uterine Wt

FIG. 16

Fat Androstenone (ug/g)
0
1
2
Immunocastrates
Late Castrates
Boars
Barrows

FIG. 17

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/CA 97/00559

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C12N15/31 C12N15/62 C12N1/21 C07K14/285
C07K7/23 A61K38/09 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 08290 A (UNIV SASKATCHEWAN) 29 April 1993 cited in the application see the whole document ---	1-24
A	WO 92 03558 A (POTTER ANDREW ;CAMPOS MANUEL (CA); HUGHES HUW P A (CA)) 5 March 1992 cited in the application see the whole document ---	1-24
A	WO 93 21323 A (UNIV SASKATCHEWAN) 28 October 1993 see the whole document ---	1-24

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1997	09. 12. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/CA 97/00559

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91 15237 A (UNIV SASKATCHEWAN) 17 October 1991 cited in the application see the whole document ---	1-24
P,X	WO 96 24675 A (UNIV SASKATCHEWAN) 15 August 1996 see page 6, line 2 - line 9; claims 1-14 -----	1-7, 10-18, 21-24

1

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/CA 97/00559

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 11,24 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat Application No
PCT/CA 97/00559

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9308290 A	29-04-93	US 5422110 A	06-06-95
		AU 2699192 A	21-05-93
WO 9203558 A	05-03-92	US 5238823 A	24-08-93
		AU 655556 B	22-12-94
		AU 8395991 A	17-03-92
		CA 2089753 A	23-02-92
		EP 0550453 A	14-07-93
		US 5594107 A	14-01-97
		US 5273889 A	28-12-93
WO 9321323 A	28-10-93	AU 3883893 A	18-11-93
		CA 2133441 A	28-10-93
		EP 0635055 A	25-01-95
WO 9115237 A	17-10-91	AT 157258 T	15-09-97
		AU 642650 B	28-10-93
		AU 5662190 A	30-10-91
		CA 2014033 A,C	07-10-90
		DE 69031351 D	02-10-97
		EP 0527724 A	24-02-93
		JP 5508301 T	25-11-93
		US 5476657 A	19-12-95
WO 9624675 A	15-08-96	AU 4477796 A	27-08-96